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PRINCIPAL INVESTIGATOR: Stephen P. Ethier, Ph.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, MI 48109

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Ann Arbor, MI 48109

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We sought to assess the importance of cooperative interactions between p185^{erbB-2} and *erb*B-3 in growth factor-independent proliferation and the neoplastic transformation of breast carcinoma cells with c-*erb*B-2 gene amplification. To emulate the process in genetically engineered non-transformed mammary epithelial cells that co-express c-*erb*B-3, MCF-10A cell populations were derived that overexpress c-*erb*B-2 at very high levels comparable to that seen in breast carcinoma cells with c-*erb*B-2 gene amplification. While the previously engineered clones of MCF-10AerbB-2 cells overexpress c-*erb*B-2 at only moderate levels, the selection of cells using Flow Cytometry with anti-p185^{erbB-2} antibody gave rise to very high-level p185^{erbB-2}-overexpressing cell populations, and further passage in the complete absence of growth factors results in the selection of cells expressing the highest level of p185^{erbB-2}. These and the other cell lines presently under construction will allow us to study the constitutive activation of PI 3-kinase, growth factor independence in culture, and the *in vivo* transformation of human mammary epithelial cells that co-express c-*erb*B-3. The other major focus of this project involves the construction of cell lines expressing dominant negative forms of c-*erb*B-2 and c-*erb*B-3. The use of the expression vectors presently under construction will allow us to test the potential of this approach for blocking p185^{erbB-2}/*erb*B-3 heterodimer function.

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Stephen V. Ehlers 6-23-97

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INTRODUCTION

We sought to assess the importance of the cooperative interactions between p185^{erbB-2} and *erb*B-3 in growth factor-independent proliferation and neoplastic transformation of breast carcinoma cells with c-*erb*B-2 gene amplification. The c-*erb*B-2 (*neu*/HER-2) gene encodes an 185 kDa protein tyrosine kinase that is highly homologous to *erb*B-1 (EGFR), *erb*B-3 and *erb*B-4 (1-3), which together, comprise the type I family of tyrosine kinase growth factor receptor genes (4, 5). The *erb*B receptor tyrosine kinases all contain ectodomains with two cysteine-rich sequences. Despite this structural homology, these receptors differ in their ligand specificities (4). Thus, EGFR binds several distinct ligands (e.g. transforming growth factor-α, amphiregulin, heparin-binding EGF and betacellulin) whose prototype is EGF, whereas *erb*B-3 and *erb*B-4 are the respective low and high affinity receptors for more than a dozen different isoforms of the *neu* differentiation factor/heregulins (HRGs) (6-8). While no direct ligand for p185^{erbB-2} has yet been cloned, it is now clear that p185^{erbB-2} is capable of heterodimerization with EGFR (9, 10), *erb*B-3 (11) or *erb*B-4 (8), and these p185^{erbB-2}-containing heterodimers form the highest affinity binding sites for their respective ligands (10, 11). c-*erb*B-2 is amplified in 28% of primary human primary breast carcinomas *in vivo* (12) and another 10% overexpress c-*erb*B-2 without amplification of the gene (14-16). In addition, c-*erb*B-2 gene amplification, concordant with high-level overexpression, is correlated with increased tumor aggressiveness and the poor prognosis of breast cancer patients (13, 14, 17-20). Other related genes, such as the EGFR gene, are sometimes amplified in human breast cancers (13). However, amplification of the EGFR gene is much less common than that seen for c-*erb*B-2 (2% vs 28%, respectively) in breast cancer. Amplification of c-*erb*B-3 or c-*erb*B-4 was not ever seen in various studies (2, 3). However, our own work combined with others have now shown that heterodimer interactions between p185^{erbB-2} and *erb*B-3 are constitutively activated in breast cancer cells which have amplified c-*erb*B-2 and co-express c-*erb*B-3 (21-24). These heterodimer complexes activate the mitogen-activated protein (MAP)-kinase and phosphatidylinositol (PI) 3-kinase signal transduction pathways. By constitutively activating key signal transduction pathways to a level that is effective for transformation, tumor cells escape the normal controls on cell cycle regulation. However, much recent evidence also suggests that a "critical threshold" level of c-*erb*B-2 gene overexpression is required to effectively transform cells. We are particularly interested in how high-level c-*erb*B-2 gene expression combined with the cooperative effects of p185^{erbB-2}/*erb*B-3 heterodimer interactions constitutively activate signal transduction pathways that transform human mammary epithelial cells.

Experimentally elevated c-*erb*B-2 gene expression in various cell lines (24-27), including non-transformed human mammary epithelial cells (27), induces the complete transformation of cells injected into nude mice. The potent oncogenic potential of p185^{erbB-2} is generally thought to be due to its ability to constitutively activate key signal transduction pathways that are involved in the regulation of cell growth. While our current understanding of the oncogenic potential of p185^{erbB-2} has expanded quite rapidly

(for review see 28), our knowledge of how exactly p185$^{erbB\text{-}2}$ induces full neoplastic transformation in human mammary epithelial cells still remains fragmentary. For example, although p185$^{erbB\text{-}2}$ was originally discovered as the *neu* transmembrane-mutated form of the gene in rat neuroblastoma cells (29), the c-*erb*B-2 gene found in human breast cancer cells has not ever shown similar mutations (30). The level of c-*erb*B-2 gene expression seen in primary human mammary carcinomas *in vivo* always shows a direct correspondence with the level of tyrosine-phosphorylated p185$^{erbB\text{-}2}$ (31), suggesting that overexpression of wild-type c-*erb*B-2 alone is sufficient to constitutively activate its tyrosine kinase function. The protein encoded by the wild-type c-*erb*B-2 gene was previously shown to exhibit constitutive tyrosine kinase activity if sufficiently overexpressed in a variety of cell lines in culture in the absence of any identifiable ligand (24-27, 32, 33). Also, transfection of a gene encoding a chimeric receptor containing the EGFR extracellular domain fused to the cytoplasmic domain of p185$^{erbB\text{-}2}$ results in the constitutive tyrosine kinase activity of the chimeric receptor in the absence of EGF (32, 33), indicating that the tyrosine kinase domain of p185$^{erbB\text{-}2}$ exhibits a greater tendency towards ligand-independent activation than does the EGFR tyrosine kinase domain when overexpressed in various cell types. Therefore, the constitutive activation of p185$^{erbB\text{-}2}$ seen in breast carcinoma cells is generally thought to be due to the high-level overexpression of the wild-type protein, whose activity shows a strong propensity towards ligand-independent activation when overexpressed (28, 32, 33).

Another area of great importance concerns the heterodimeric associations which have been recently shown to occur between the different type 1 receptor tyrosine kinases, including EGFR and p185$^{erbB\text{-}2}$ (9, 10), p185$^{erbB\text{-}2}$ and *erb*B-3 (11, 12), p185$^{erbB\text{-}2}$ and *erb*B-4 (8), and EGFR and *erb*B-3 (34, 35). These heterodimeric interactions may occur through both ligand-dependent and ligand-independent mechanisms. Activation of tyrosine kinase activity of these receptors involves dimerization, trans-autophosphorylation and the recruitment of various signal transduction molecules by specific tyrosine-phosphorylated receptor carboxyl-terminal sites (that are different for the various *erb*Bs). Therefore, differential responses mediated by the various receptors and their respective ligands can become quite complex, both because of the various combinatorial associations between the different receptors, and the relative affinities between different receptor dimers at various levels of expression. The possible interactions between the different *erb*B receptors has only very recently emerged as an important concept in *erb*B-2-related cancer biology. As mentioned above, our own work combined with others has now shown that the heterodimer interactions between p185$^{erbB\text{-}2}$ and *erb*B-3 are constitutively activated in breast cancer cells with c-*erb*B-2 gene amplification (21-24). The cooperative interactions between the different receptor types may help explain the pleiotropic effects of p185$^{erbB\text{-}2}$ on multiple signaling pathways which leads to the full transformation of cells. Furthermore, studying these interactions may eventually lead to a better understanding of new potential targets for clinical intervention employing gene therapy and other methods.

Our laboratory is interested in studying mechanisms of growth factor dependency in mammary epithelial cells. We have previously shown that the growth

factor-independent proliferation of mammary carcinoma cells in culture is strictly associated with their malignant potential when serially transplanted *in vivo* (36). Both autocrine (37) and non-autocrine (38) mechanisms may be involved in these phenomena, depending on the particular tumor and stage of the disease. Other studies have shown a correlation of growth factor-independent proliferation in culture with tumor metastasis *in vivo* in different systems (39). Therefore, growth factor independence, as a phenotype, is a particularly good indicator of progressive cell transformation in tumor cells. Normal human mammary epithelial cells require both insulin-like growth factor (IGF)-I (or supra physiological levels of insulin) and epidermal growth factor (EGF) to proliferate under serum-free conditions in culture (40-42). The synergistic requirement for both IGF and EGF during the mitogen-dependent proliferation of normal mammary epithelial cells suggests that the attainment of growth factor-independent proliferation in mammary carcinoma cells involves genetic changes which subvert the normal requirements for both IGFs and EGF during mammary epithelial proliferation. We have recently shown that 21MT human breast carcinoma cell lines, that overexpress progressively elevated levels of c-*erb*B-2 (see original proposal), exhibit IGF independence at moderate levels of c-*erb*B-2 overexpression, and combined IGF/EGF independence at the highest level of c-*erb*B-2 overexpression (43). Furthermore, we found that the HRGs are mitogenic for human mammary epithelial cells (which express both c-*erb*B-2 and c-*erb*B-3, but not c-*erb*B-4) in the absence of IGF and EGF in culture (44). Thus, heregulins mimic the combined actions of both IGF and EGF in mammary epithelial cells. This indicates that the combined activation of $p185^{erbB-2}$ and *erb*B-3 mediates the activation of signal transduction mechanisms which substitute for IGF and EGF stimulation of mammary epithelial cell proliferation. The ability of various growth factors to stimulate normal cell growth was previously shown to fall into two different functional categories termed competence (e.g. EGF) and progression (e.g. IGF-1) factors (45). EGF is required during the early part of the G1 phase of the cell cycle to make the cells competent for proliferation, while IGF is required towards the end of G1 for the cell to progress into S phase of the cycle, and both EGF and IGF are required transiently during the middle of G1 (45). Therefore, these different growth factors act synergistically to stimulate proliferation in a fashion that is not simply additive, but is indicative of synergism between growth factor responsive pathways which must be activated together for mitogenesis in non-transformed cells that express normal receptor levels. Interestingly, much of the work concerned with understanding how oncogene expression subverts these growth factor requirements in tumor cells has emphasized responses associated with the requirement for competence factors, because other oncogenes, such as v-*erb*B (a truncated form of EGFR), were only implicated in the activation of progression pathways that normally require EGF for stimulation (46). We propose that $p185^{erbB-2}$ constitutive activation in mammary carcinoma cells substitutes for growth factor-mediated signal transduction pathways normally requiring the combination of both IGF and EGF, and this autonomous growth potential depends on the level of $p185^{erbB-2}$ activation in specific tumor cell populations as well as its interaction with other *erb*B receptors. These

properties of the *erb*B-2 protein may make it a particularly good target for the malignant transformation of breast cancer cells.

Our data, in addition to others, now strongly implicate PI 3-kinase-mediated signal transduction in HRG-induced mitogenesis and the autonomous growth of human mammary carcinoma cells with c-*erb*B-2 gene amplification (22, 23). However, further work is required to determine the minimum level of c-*erb*B-2 overexpression that is required for constitutive PI 3-kinase activation, growth factor independence and tumorigenicity in mammary epithelial cells that co-express low, but functional levels of c-*erb*B-3. Previous studies have shown that experimentally elevated c-*erb*B-2 gene expression in non-neoplastic human mammary epithelial cells is sufficient to convert the cells to a full neoplastic phenotype when injected into nude mice (27). Interestingly, the growth of the transfected cell populations in nude mice showed much greater elevation of c-*erb*B-2 gene expression in tumor cells which grew out *in vivo* then did the original transfectants, and this higher level of c-*erb*B-2 overexpression correlated with increased soft agarose growth in culture and increased tumor growth *in vivo* in a manner which suggests that the selection of variants containing higher levels of c-*erb*B-2 expression was required to induce full transformation (27). In addition, earlier it was reported that c-*erb*B-2 overexpression alone was not sufficient to fully transform MCF-10A cells (47). However, these MCF-10erbB-2 cell clones only showed moderate levels of c-*erb*B-2 gene expression relative to tumor cells with c-*erb*B-2 gene amplification (unpublished results; see below). Additionally, a cell line derived from the primary tumor in the same patient as the 21MT metastatic breast carcinoma cells (48, 49) have equivalently amplified the c-*erb*B-2 gene, but show much lower levels of gene expression and constitutive $p185^{erbB-2}$ activation then do the 21MT cell lines (manuscript in preparation). The 21PT cells do not show a high level of growth factor independence in culture and do not form tumors in nude mice (49). This also supports the contention that there is a critical threshold level of c-*erb*B-2 overexpression (i.e. between that seen in 21PT cells and in 21MT cells) that is necessary to effectively transform mammary epithelial cells. The minimum level of c-*erb*B-2 overexpression that is required for full transformation in mammary epithelial cells (that co-express c-*erb*B-3) has not yet been exactly determined. In addition, none of the above studies with transfected mammary cells have reported any of the signal transduction events which underlie these phenomena in genetically engineered human mammary epithelial cells. Therefore, we have begun a series of experiments to address these questions by producing c-*erb*B-2-overexpressing mammary epithelial cell lines by infection with retroviral expression vectors combined with procedures for the selection of cell populations that overexpress progressively higher levels of $p185^{erbB-2}$. Using these cell lines, we can now directly address our objectives to determine the critical level of c-*erb*B-2 overexpression and constitutive activation that is necessary to effectively transform various normal human mammary epithelial cell lines.

In this project, we have also focused on specific strategies for blocking $p185^{erbB-2}$/*erb*B-3 interactions in breast carcinoma with c-*erb*B-2 gene amplification. We have begun to develop new expression vectors for blocking $p185^{erbB-2}$ and *erb*B-3 function in our cell lines. The relative importance of the $p185^{erbB-2}$/*erb*B-3 interaction

(relative to p185$^{erbB\text{-}2}$ homodimer function) in the constitutive activation of PI 3-kinase and the growth factor independence of the 21MT cells has not yet been clearly established. One strategy which has been used successfully for specifically blocking receptor function involves the use of dominant negative expression vectors, wherein the region coding for the cytoplasmic region of the receptor is deleted (see original proposal). While the mutant receptor can still dimerize with wild-type receptors within the cell, it lacks kinase activity and signal transducer docking function. Therefore, by specifically removing this region the receptor function is completely impaired. This strategy has been used effectively for blocking the EGFR (50), platelet-derived growth factor β receptor (51), fibroblast growth factor receptor (52), and *neu* (53) function in various studies. In order to block the HRG-induced activation of p185$^{erbB\text{-}2}$ and *erb*B-3 in mammary epithelial cells, as well as the constitutive activation of these receptors in the 21MT mammary carcinoma cells, we have begun to construct dominant negative vectors coding for p185$^{erbB\text{-}2}$ and *erb*B-3 for introduction into the various mammary epithelial and carcinoma cell lines. The introduction of these dominant negative vectors will allow us to better assess the importance of p185$^{erbB\text{-}2}$/*erb*B-3 heterodimers in the autonomous growth of carcinoma cells with c-*erb*B-2 gene amplification.

BODY

In order to determine the level of c-*erb*B-2 gene overexpression that is required for mammary epithelial cells to constitutively activate PI 3-kinase, induce growth factor independence and transform mammary epithelial cells (i.e. Specific Aims 1 and 3), it is necessary to genetically engineer human mammary epithelial cell lines that overexpress c-*erb*B-2 at very high levels comparable to that seen in breast carcinoma cells with c-*erb*B-2 gene amplification. As discussed above, previous studies involving genetically engineered MCF-10A cells were not successful in generating cell populations which sufficiently overexpress c-*erb*B-2 at levels comparable to that seen in breast carcinoma cells with c-*erb*B-2 gene amplification. Thus, it is of primary importance to develop new p185$^{erbB\text{-}2}$-overexpressing MCF-10A and H16N-2 cell lines for a more critical assessment of threshold levels of c-*erb*B-2 gene overexpression required for transformation in human mammary epithelial cells that co-express c-*erb*B-3. To accomplish the initial specific aims of this project, we constructed a bicistronic retroviral vector containing the c-*erb*B-2 gene under the control of the CMV promoter (see original proposal). We also utilized previously constructed cell lines which overexpress c-*erb*B-2 to select for high-level overexpressing cell populations using Flow Cytometry (see below). The original pSLHerbB-2 vector we produced was constructed by blunt-end ligation of the full-length c-*erb*B-2 cDNA into the SLH1001 retroviral expression vector. However, transfection and infection of cell lines with this vector did not show increased expression of c-*erb*B-2 (data not shown). We have previously used the SLH1001 vector containing the lac Z gene successfully. Therefore, it is likely that alterations in the ends of the c-*erb*B-2 cDNA or the SLH1001 vector may have occurred during the blunt-end ligations that

prevented proper expression. More recent strategies for constructing the required c-*erb*B-2 bicistronic vector now involve the insertion of the c-*erb*B-2 cDNA into the pBK-CMV phagemid expression vector (Stratagene) as an intermediary step followed by directional cloning of c-*erb*B-2 into the SLH1001 vector in a more straightforward manner not involving blunt-end ligation. The newly constructed vector will be invaluable for infection of various cell lines that will be used for studying the effects of $p185^{erbB-2}$/*erb*B-3 on mammary epithelial cell transformation. During the course of this work, we had also employed alternate strategies to derive c-*erb*B-2-overexpressing MCF-10AerbB-2 cell lines that overexpress c-*erb*B-2 at very high levels comparable to that seen in breast carcinoma cells with c-*erb*B-2 gene amplification. Interestingly, while the original MCF-10AerbB-2 cells do not show sufficiently high levels of c-*erb*B-2 expression, by using Flow Cytometry and other selection strategies involving growth factor deprivation we have now successfully generated MCF-10AerbB-2 cell lines showing very high levels of c-*erb*B-2 gene overexpression.

We and others have previously used the MCF-10A normal human mammary epithelial cell line engineered to overexpress c-*erb*B-2 (44, 47). The parental MCF-10A cell line was isolated from normal reduction mammoplasty tissue, and is not tumorigenic in nude mice (54). MCF-10A cells were previously infected with a retroviral expression vector containing the human c-*erb*B-2 gene (Fig. 1; 47). As mentioned above, we have recently confirmed that MCF-10AerbB-2 cells do express elevated levels of constitutively tyrosine-phosphorylated $p185^{erbB-2}$ (44). However, they overexpress c-*erb*B-2 at only a fraction of what is measured in mammary carcinoma cells with c-*erb*B-2 gene amplification (unpublished data), and they show very little growth factor independence in culture (44). Although $CdCl_2$ or $ZnCl_2$ was added to cultures in various experiments, the metallothionine promoter that drives c-*erb*B-2 expression in the pN02-erbB-2 vector was found to be very leaky and showed no significant induction in the presence of heavy metals (data not shown). More recently, however, we noticed a wide range of expression levels of cell surface $p185^{erbB-2}$ in MCF-10AerbB-2 cells in experiments utilizing Flow Cytometry to measure cell surface $p185^{erbB-2}$ with FITC-conjugated antibodies directed against $p185^{erbB-2}$ (Fig. 2). Cell populations comprising the highest 5% of c-*erb*B-2 overexpressing cells were selected and then passaged for later analysis (Fig. 2; i.e. channel "C"). Other selection procedures were also employed in an effort to select cells with higher levels of c-*erb*B-2 gene overexpression in these heterogenous clones from the original retroviral-infected MCF-10AerbB-2 cell population. Other strategies for selecting higher-level expression of c-*erb*B-2 involved increasing the selective pressure with titrations of higher levels of hygromycin, as well as by the selection of growth factor-independent phenotypes in culture (Table 1).

Flow Cytometry selection of MCF-10AerbB-2 cell populations with antibodies to surface $p185^{erbB-2}$ was quite effective in deriving a population of cells with significantly elevated $p185^{erbB-2}$ that is comparable to that seen in the 21MT breast carcinoma cells with c-*erb*B-2 gene amplification (Fig. 3 and 4). Interestingly, exposure to high levels of hygromycin actually selected for a lower level of c-*erb*B-2 expression (Fig. 4). Thus,

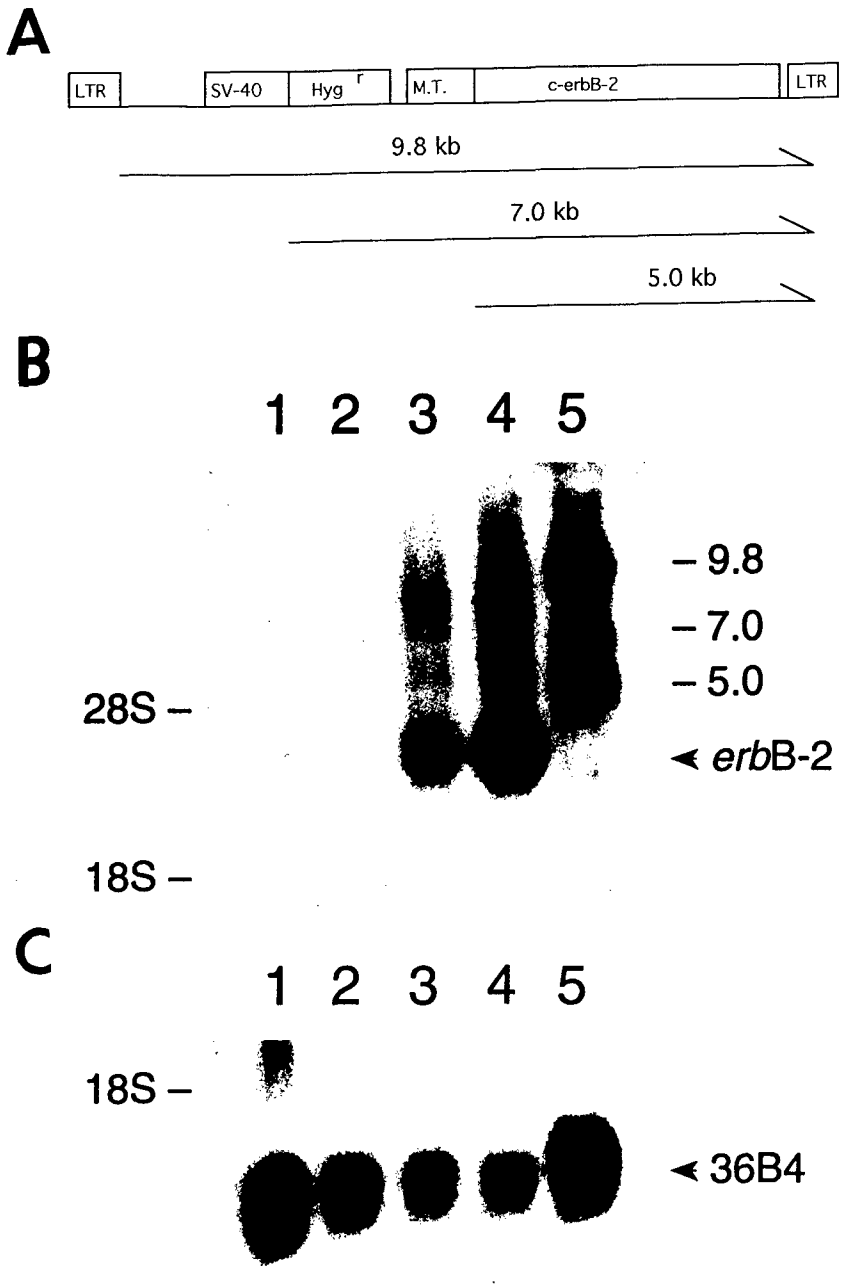

Fig. 1: ***MCF-10A cells previously infected with the pN02-erbB-2 vector.*** Diagram of the pN02-erbB-2 vector (A) previously used to infect the normal MCF-10A human mammary epithelial cell line. Northern blot analysis of c-*erb*B-2 (B) and 36B4 (C) gene expression in MCF-10A (Lane 1), H16N-2 (Lane 2), 21MT-2 (Lane 3), 21MT-1 (Lane 4), and MCF-10AerbB-2 (Lane 5) cells. The H16N-2 cell line was also derived from normal tissue, while the 21MT-2 and 21MT-1 cell lines were isolated from a pleural effusion taken from the same patient as the H16N-2 cells, and have amplified c-*erb*B-2.

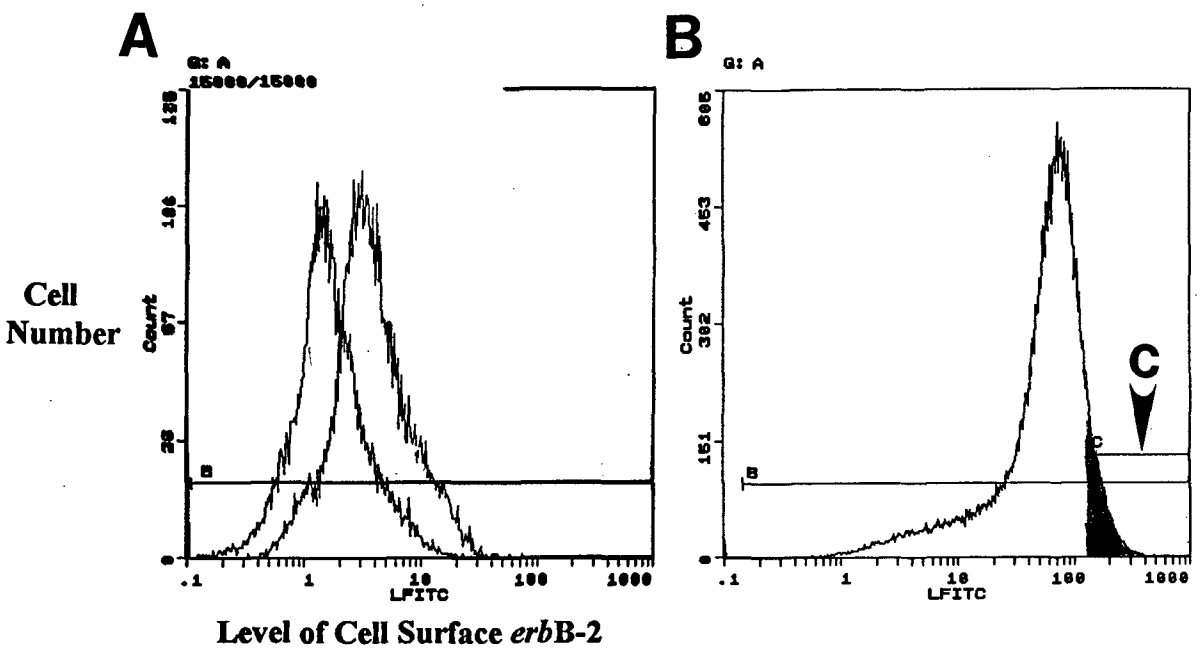

Fig. 2: ***Flow Cytometry of p185^{erbB-2} levels in MCF-10AerbB-2 cells.*** Control cells (A) or MCF-10AerbB-2 cells (B) were labeled with an anti-p185^{erbB-2} monoclonal antibody (Tab 254)/FITC-conjugated secondary antibody, and then sorted through a Flow Cytometer in order to collect the top 5% of c-*erb*B-2 overexpressing cells (i.e. channel C). These cells, designated MCF-10AerbB-2sh, were then passaged for further analysis and selection strategies.

Table 1: ***Derivation of the MCF-10AerbB-2 cell line series********.

Cell line	MCF-10A	MCF-10A erbB-2	MCF-10A erbB-2 hyg400	MCF-10A erbB-2sh	MCF-10A erbB-2shH
Origin	Normal reduction mammoplasty tissue	MCF-10A cells infected with pNO2-erbB-2 vector	MCF-10AerbB-2 cells selected on high levels hygromycin	MCF-10A erbB-2 cells after Flow Cytometry	MCF-10A erbB-2sh cells cultured w/o growth factors
Infected with vector	no	yes	yes	yes	yes
c-*erb*B-2 gene expression	low	low-moderate	low	moderate-high	high

* See text for details of derivation for these new c-*erb*B-2-overexpressing cell lines.

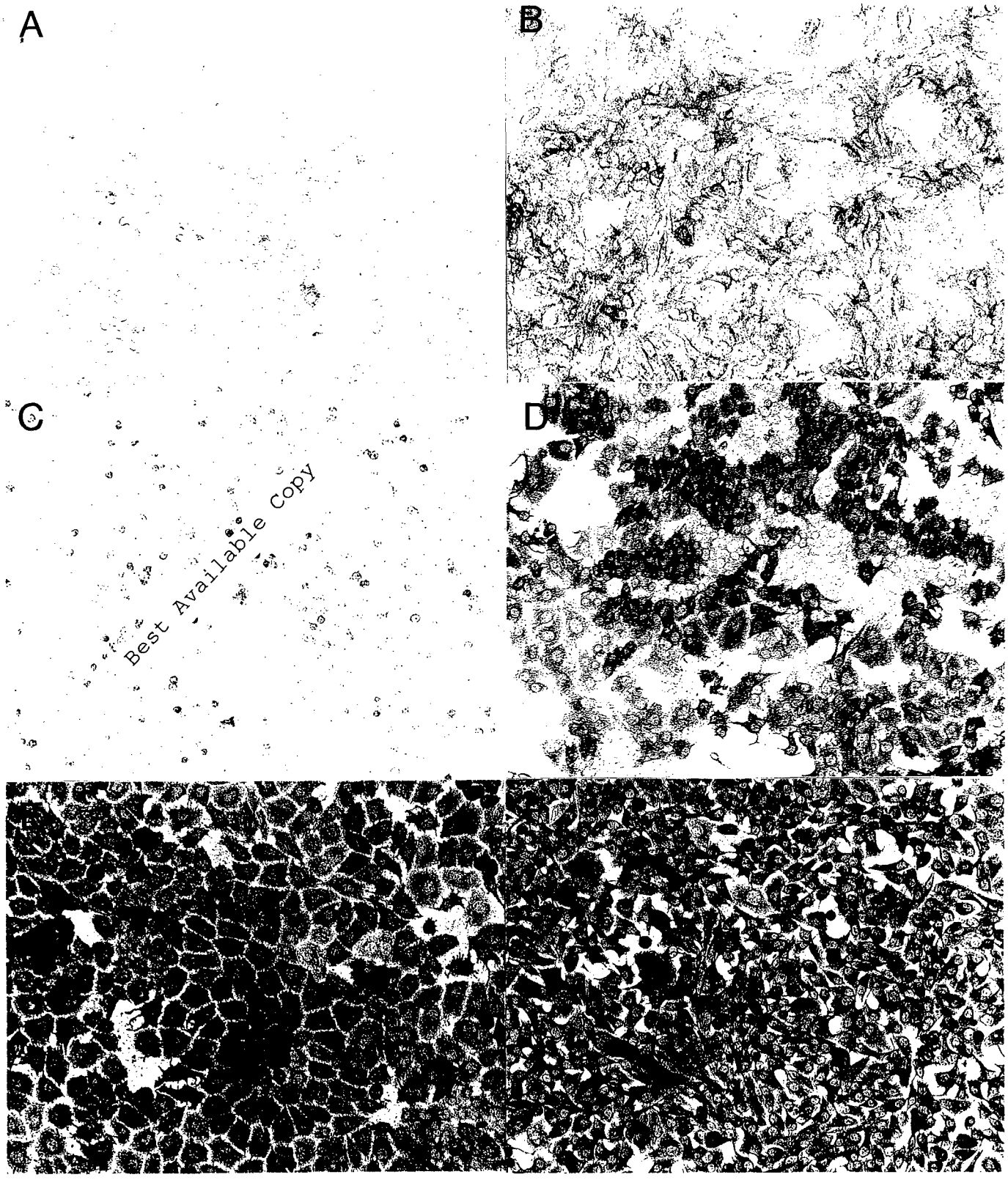

Fig. 3: ***Expression of p185^{erbB-2} in variously selected MCF-10AerbB-2 cell populations in culture.*** Immunocytochemistry of cells labeled with anti-p185^{erbB-2} monoclonal antibody (Tab 254) followed by strep-avidin/HRP detection with diaminobenzidine. MCF-10A cells (A). MCF-10AerbB-2 cells (B). MCF-10AerbB-2hyg400 cells (C). MCF-10AerbB-2sh cells (D). MCF-10AerbB-2shH cells (E). 21MT-1 cells (F).

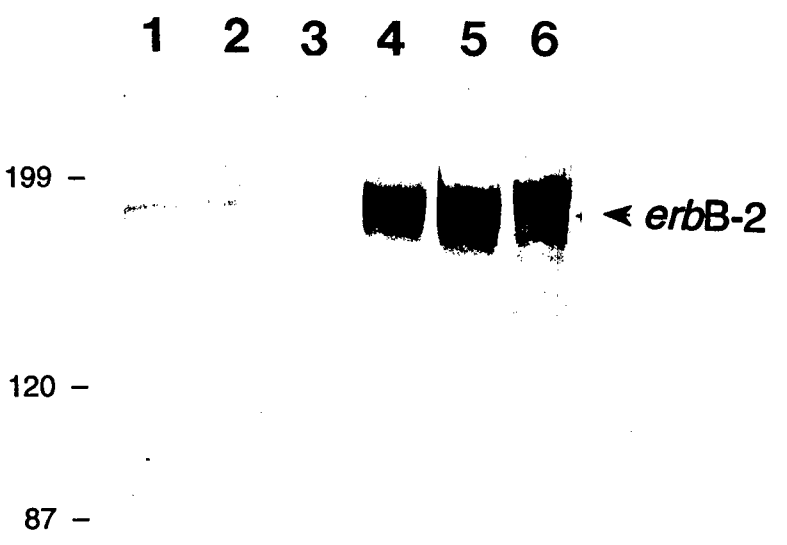

Fig. 4: ***Expression of p185^{erbB-2} in variously selected MCF-10AerbB-2 cell populations in culture.*** Western blots containing equal (100 ug) lysate protein per lane were probed with anti-p185^{erbB-2} polyclonal antibody (9.3) and visualized by strep-avidin/HRP detection with diaminobenzidine and nickel chloride. MCF-10A cells (Lane 1). MCF-10AerbB-2 cells (Lane 2). MCF-10AerbB-2hyg400 cells (Lane 3). MCF-10AerbB-2sh cells (Lane 4). MCF-10AerbB-2shH cells (Lane 5). 21MT-1 cells (Lane 6).

hygromycin actually selected for a lower level of c-*erb*B-2 expression (Fig. 4). Thus, rather than showing coordinate co-expression of high-level antibiotic resistance with elevated p185^{erbB-2}, antibiotic resistance and p185^{erbB-2} levels are inversely related. This is not too surprising when considering the structure of the original pN02-erbB-2 vector used to infect these cells (Fig. 1), because of the dual promoter competition phenomena that has been previously shown to occur in retroviral vectors containing multiple promoters (55). Thus, you would expect to see inversely related expression of the antibiotic-resistance gene with the gene of interest (i.e. c-*erb*B-2), because by selecting for the expression of one gene on the vector you drive down the expression of the other. Also keep in mind that the three different nested transcription units coded for by the pN02-erbB-2 vector contain no internal ribosome initiation sites, and thus only the smallest 5 kb transcript would allow efficient translation of the c-*erb*B-2 gene. This can be directly tested by Northern blot analysis, where the smaller transcript would be expected to show an inverse relationship with the middle band (which contains the functional transcript for hygromycin resistance). These Northern blotting experiments are presently underway, as well as Southern blotting to determine integrated vector copy number in the various MCF-10AerbB-2-derived cell lines. In contrast to that seen with selection with high levels of hygromycin, growth factor deprivation conditions increase the levels of p185^{erbB-2} above that seen with Flow Cytometry selection alone (Fig. 4). The levels of activated p185^{erbB-2} in both Flow Cytometry-selected cell populations, MCF-10AerbB-2sh and MCF-10AerbB-2shH cells, was considerably elevated over that seen in the original MCF-10AerbB-2 cell population as shown in anti-phosphotyrosine blots (data not shown).

Also, immunocytochemistry of p185$^{erbB\text{-}2}$ levels in the variously selected cell populations indicate that selection of growth factor-independent phenotypes by growth factor deprivation leads to a more homogenous population of very high-level c-*erb*B-2-overexpressing cells (Fig. 3). Dramatic alterations in cell morphology were also observed for the MCF-10AerbB-2sh and MCF-10AerbB-2shH cell lines when compared to the MCF-10AerbB-2 cells (data not shown), possibly indicative of a more fully transformed phenotype. We now intend use these cell lines for further in-depth study of the effects of progressively elevated c-*erb*B-2 gene overexpression on constitutive signal transduction, growth factor independence in culture, and tumorigenicity *in vivo*.

One interesting observation that was made following the Flow Cytometry selection of MCF-10AerbB-2 cells. It was noticed that, while MCF-10A and MCF-10AerbB-2 cells die off in the absence of all growth factors in culture, many MCF-10Ash cells survive after the withdrawal of all growth factors under serum-free culture conditions (Fig. 5). This then allowed us to culture these cells for an extended period in the complete absence of growth factors under high-density conditions (Table 1). After confluent cultures were left a month in the absence of all growth factors, these cells began to develop foci-like structures (Fig. 5C), and these cultures were then passaged at a low split ratio in the absence of any growth factors. As mentioned above, these MCF-10AshH cells selected in the absence of growth factors show a more homogenous high-level c-*erb*B-2 expression than do the original Flow Cytometry-selected MCF-10AerbB-2sh cell population (Fig. 3). This suggests that the selection of these c-*erb*B-2-overexpressing cells in the absence of growth factors results in the preferential survival of the highest-level overexpressing cells within the original cell population. Both MCF-10AerbB-2sh and MCF-10AerbB-2shH cells show a very high level of IGF-independent growth in culture (data not shown). While MCF-10AerbB-2shH cells grow slowly in the absence of EGF under high density culture conditions, preliminary data indicate that these cells still only show low levels of EGF-independent growth under low density conditions in culture (data not shown). Preliminary data indicate that PI 3-kinase activation in both flow cytometry-selected cell populations is significantly elevated (data not shown), and further immunoprecipitation/Western blot analysis is underway to directly compare the constitutive activation of PI 3-kinase in these cells with that seen in the 21MT breast carcinoma cell lines. In addition, experiments are underway to determine the tumorigenic potential of these newly derived MCF-10AerbB-2sh and MCF-10AshH cell lines in nude mice in relation to the level of c-*erb*B-2 gene overexpression and the constitutive activation of p185$^{erbB\text{-}2}$, *erb*B-3 and PI 3-kinase.

In order to accomplish the additional specific aims for this project, it is necessary to construct dominant negative vectors for c-*erb*B-2 and c-*erb*B-3 (see original proposal). Constructing the dominant negative vector for c-*erb*B-3 is an especially important focus for this project, because this has not been previously attempted and it may be especially effective in blocking the heterodimer interactions between p185$^{erbB\text{-}2}$ and *erb*B-3. As originally outlined in the project proposal, we attempted to clone PCR-generated fragments of c-*erb*B-2 and c-*erb*B-3 cDNAs into the SLH1001 bicistronic retroviral vector. While the amplification of the 2 kb regions of reverse-transcribed mRNAs was

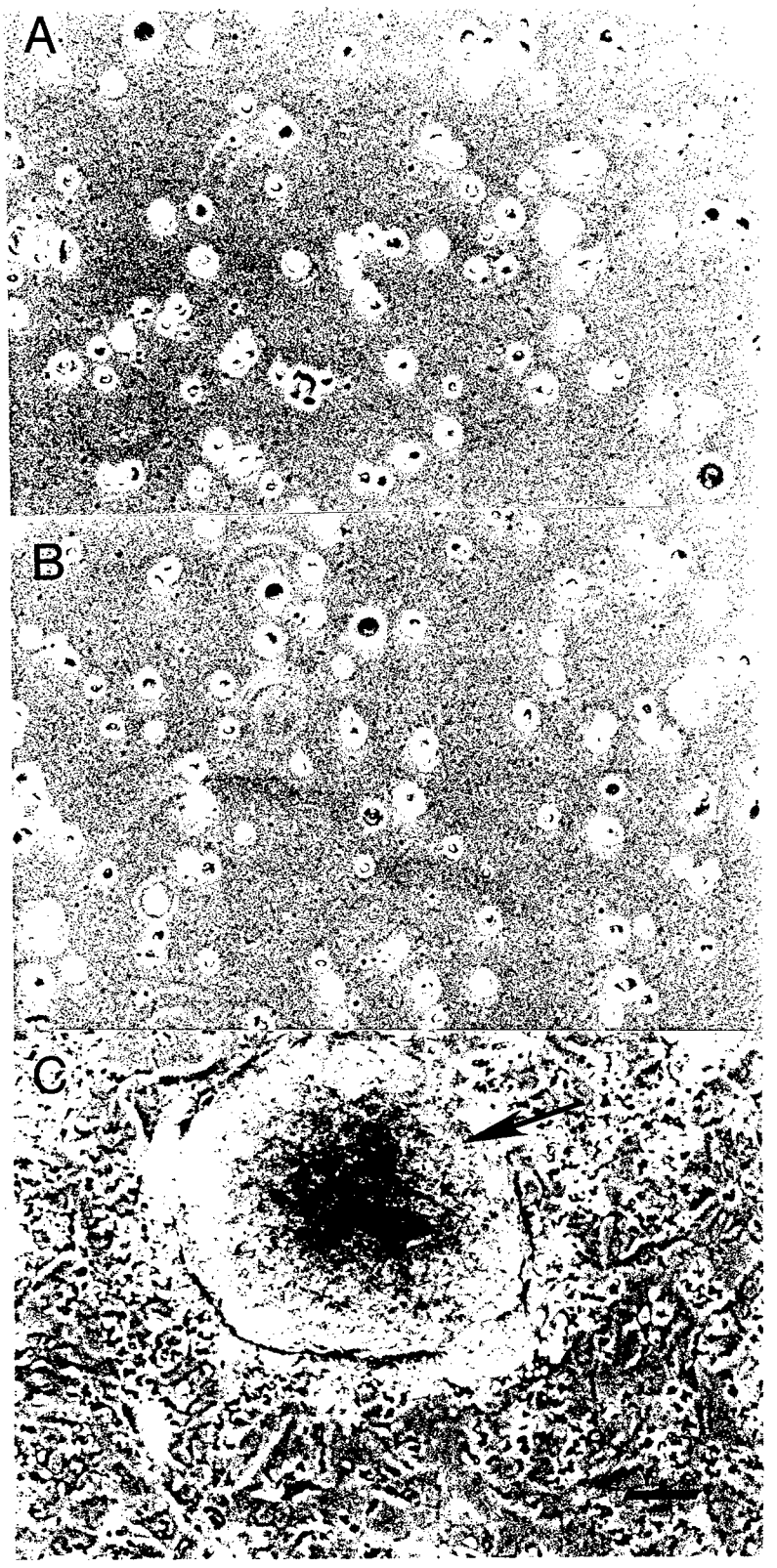

Fig. 5: ***MCF-10AerbB-2sh cells survive in the absence of growth factors in culture.*** Phase contrast microscopy of MCF-10A (A), MCF-10AerbB-2 (B), and MCF-10AerbB-2sh (C) cells after long-term growth factor deprivation in serum-free culture. MCF-10AerbB-2sh survive in the absence of any growth factors and form foci-like structures after long-term growth factor deprivation in high density culture (arrow). Bar = 100 uM.

accomplished successfully using a high-fidelity PCR kit (Bohringer Mannheim), these fragments could not be ligated into the Xho I and Cla I restriction sites in the SLH1001 vector. The primers used to generate these fragments were designed to also contain compatible sites (i.e. Sal I and Cla I) in the proper orientation for ligation into the SLH1001 vector (see original proposal). However, during the course of these experiments, it was noted that Sal I sites (that are compatible with Xho I in SHL1001) are known to be only inefficiently cleaved when located in the terminal region of linear DNA fragments. Therefore, we attributed the inability to derive ligation products in these reactions (which also included positive control reactions) to the lack of proper restriction digestion of the PCR-generated fragments prior to ligation. More recently, we have successfully introduced PCR-generated fragments coding for dominant negative forms of *erb*B-2 and *erb*B-3 into SLH1001 using alternate primers. However, we have not yet been able to detect the expression of the dominant negative proteins in recipient cell lines (data not shown). One series obstacle to strategies for constructing expression vectors with PCR-generated fragments may involve the deleterious mutations that occur at a significant frequency in amplified products, even in protocols using high-fidelity Pou/Taq polymerase enzyme combinations.

An alternate approach for developing the dominant negative expression vectors has now been devised using the available full-length cDNAs (which were only more recently available for c-*erb*B-3). By using the pBK-CMV phagemid expression vector (Stratagene) as an intermediate, we will be able to clone dominant negative inserts for c-*erb*B-2 and c-*erb*B-3 into the SLH1001 bicistronic retroviral expression vector using flanking restriction sites located within the extensive polylinker region of pBK-CMV. When the cDNAs that we have for c-*erb*B-2 and c-*erb*B-3 are cleaved with Hind III/Bal I and Sal I/Bam HI, respectively, 2.4 kb and 2.2 kb fragments lacking most of the cytoplasmic domains of these genes will be generated that will then be ligated into Hind III/Sca I and the Sal I/Bam HI sites in pBR-CMV. These ligations will also introduce in-frame stop codons downstream of the point of ligation. These pBR-CMV-derived dominant negative vectors will be used directly in experiments with the 21MT tumor cell lines, since the 21MT cell lines show a reasonably high transfection efficiency with antibiotic resistance and marker gene co-expression when transfected with standard monocistronic vectors (unpublished data). For introduction of the vectors into more normal cell lines (which do not show reasonably high transfection efficiencies with antibiotic resistance and marker gene co-expression when transfected with standard monocistronic vectors), newly constructed retroviral expression vectors will be required for infection of the cell lines with dominant negative expression vectors. This will be performed by ligation of Sal I/Cla I-cut inserts isolated from pBR-CMV-derived c-*erb*B-2 and c-*erb*B-3 dominant negative vectors into the Xho I/Cla I sites (i.e. Sal I and Xho I have compatible ends) of SLH1001 (see original proposal). These vectors will be invaluable for accomplishing specific aims 2 and 4 of this grant proposal. We have also begun nude mouse transplantation experiments using the 21MT cell lines in preparation for subsequent experiments utilizing cell lines infected with *erb*B-2 and *erb*B-3 dominant negative expression vectors.

CONCLUSIONS

We have now successfully produced MCF-10A-derived cell populations that overexpress c-*erb*B-2 at very high levels comparable to that seen in breast carcinoma cells with c-*erb*B-2 gene amplification. These and the other cell lines presently under construction will be invaluable in fulfilling the specific aims of this project. In addition, key observations were made during the course of these studies which provide important information concerning the survival and growth of cells in the absence of growth factors that occurs as a function of the level of c-*erb*B-2 gene overexpression. While the original clones of MCF-10AerbB-2 cells overexpress c-*erb*B-2 at only moderate levels, very high-level overexpression of c-*erb*B-2 was obtained by selecting cells from the original heterogenous MCF-10AerbB-2 cell population using Flow Cytometry with anti-$p185^{erbB-2}$ antibody. Following Flow Cytometry selection of c-*erb*B-2-overexpressing cells, these cells were then futher selected in the complete absence of growth factors in serum-free culture, and this further increased the levels of $p185^{erbB-2}$. Experiments are presently underway to measure the growth factor responsiveness and the relative level of PI 3-kinase activation for these cell lines with and without various growth factors in culture. Furthermore, nude mouse studies are underway to determine the tumorigenic potential and level of $p185^{erbB-2}$, *erb*B-3 and PI 3-kinase activation in these cell lines *in vivo*.

Interestingly, previous studies employing NIH 3T3 as a recipient cell line initially reported that the wild-type c-*erb*B-2 was not oncogenic. However, subsequent work showed that these earlier studies had not properly tested the oncogenic potential of the wild-type c-*erb*B-2 gene, because the levels of c-*erb*B-2 overexpression in these c-*erb*B-2-overexpressing cells was not high enough to constitutively activate $p185^{erbB-2}$ to levels sufficient to transform NIH 3T3 fibroblast cells (24-26). By using different promoters and the DHFR replicon in newly developed expression vectors, subsequent researchers were able to generate cell lines which overexpress very high levels of $p185^{erbB-2}$, and were able to properly test the oncogenic potential of the wild-type c-*erb*B-2 gene in NIH 3T3 cells (24-26). In those studies, it was also apparent that a level of $p185^{erbB-2}$ of approximately $5x10^5$- $8x10^6$ receptors/cell were required for transformation mediated by $p185^{erbB-2}$ homodimers (i.e. NIH 3T3 cells do not express significant levels of the other *erb*B kinases). As mentioned above, more recent studies have now also demonstrated a cooperative transforming capability *erb*B-3 with $p185^{erbB-2}$ in NIH 3T3 cells (22). However, it is still not clear how the presence of even low levels of *erb*B-3 quantitatively affects the critical threshold level of $p185^{erbB-2}$ required for transformation in human mammary epithelial cells. The studies that have tested the oncogenic potential of wild-type c-*erb*B-2 overexpression in human mammary epithelial cells are likely to be directly analogous to that seen earlier using NIH 3T3 cells, in that reports of the ability of c-*erb*B-2 to fully transform MCF-10A cells has not yet been adequately tested due to the difficulty of generating cell lines that overexpress sufficiently high levels of $p185^{erbB-2}$. We have now made major progress towards resolving this issue by deriving MCF-10AerbB-2 cells that do overexpress c-*erb*B-2 at levels comparable to that seen in breast carcinoma cells with c-*erb*B-2 gene amplification. In addition, by using cell lines which

co-express *erb*B-3, we can now directly test the effects of *erb*B-3 cooperativity in human mammary epithelial cells that overexpress high-levels of c-*erb*B-2.

Flow Cytometry-selected MCF-10AerbB-2sh cells were also found to survive in the absence of any exogenous growth factors under high density culture conditions, and these cells were then cultured for extensive periods under selective pressure in the absence of exogenous growth factors. This selective pressure was found to lead to a more homogenous distribution of high-level c-*erb*B-2 overexpressing cells. These experiments, in addition to others utilizing the 21T breast carcinoma cell lines (manuscript in preparation), further support the contention that there is an important relationship between growth factor independence and the progressively increasing levels of c-*erb*B-2 gene expression seen in breast carcinoma cells with c-*erb*B-2 gene amplification during tumor progression (43). Interestingly, it was recently reported that p53-independent apoptosis is induced in MCF-10A cells in response to IGF and EGF deprivation in culture (56). This study also showed that the actions of the MAP-kinase and PI 3-kinase pathways were required to prevent apoptosis in non-neoplastic mammary epithelial cells (56). Our results now also suggest that the constitutive activation of $p185^{erbB-2}$, *erb*B-3 and PI 3-kinase induced by high-level wild-type $p185^{erbB-2}$ overexpression promotes cell survival in the absence of exogenous growth factors in culture. Further study of the minimum level of c-*erb*B-2 overexpression required to induce these effects will allow us to better understand the pleiotropic effects of c-*erb*B-2 overexpression during mammary tumor progression. In addition, recent studies now also indicate that c-*erb*B-2 overexpression combined with p53 inactivation is correlated with poorer prognosis than c-*erb*B-2 overexpression alone in breast cancer patients (57). This may have important implications regarding the cooperative effects of tumor suppressor gene inactivation with c-*erb*B-2 gene amplification during tumor progression. As outlined in the original proposal, we are also constructing c-*erb*B-2-overexpressing H16N-2 cells. The H16N-2 cells, which were isolated from the same patient as the 21MT breast carcinoma cell lines by transfection of normal mammary epithelium with the human papilloma virus, will provide an independent test of the oncogenic potential of c-*erb*B-2 overexpression in non-neoplastic human mammary epithelial cells that co-express c-*erb*B-3 (43). Interestingly, previously we found that the H16N-2 cells are hyper responsive to the mitogenic effects of HRGs (23, 43) when compared to MCF-10A or MCF-10AerbB-2 cells (44). More recently, we found that these differences are likely due to the cooperative effects of suppressor gene inactivation in the H16N-2 cells (manuscript in preparation). Thus, by using a cell line with p53 and RB inactivation induced the E6 and E7 genes, we can compare the transforming potential of $p185^{erbB-2}$ in mammary cells with specific suppressor gene inactivation (i.e. the MCF-10A cells contain functional p53 and RB; 56). We have also recently constructed MCF-10A cells that express the E6 and E7 genes separately, and these cell lines may also prove valuable for later studies comparing the oncogenic potential of $p185^{erbB-2}$/*erb*B-3 in cells containing various alterations in specific suppressor gene functions.

The other major focus of this project involves the construction of cell lines expressing dominant negative vectors for c-*erb*B-2 and c-*erb*B-3 (see original proposal).

The strategy for vector construction now involves a method utilizing cDNAs and vector splicing that is more likely to yield functional vectors in the near future. The use of these vectors will then allow us to successfully complete the specific aims of the project involving the inhibition of p185$^{erbB\text{-}2}$/*erb*B-3 heterodimer function in breast carcinoma cells that overexpress c-*erb*B-2.

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APPENDICES

Two reprints are included.

Phosphatidylinositol 3-Kinase Recruitment by p185^{erbB-2} and *erb*B-3 Is Potently Induced by *neu* Differentiation Factor/Heregulin during Mitogenesis and Is Constitutively Elevated in Growth Factor-independent Breast Carcinoma Cells with c-*erb*B-2 Gene Amplification[1]

Tracy G. Ram and Stephen P. Ethier[2]

Department of Radiation Oncology, University of Michigan Medical School, Ann Arbor, MI 48109-0582

Abstract

Amplification and overexpression of the c-*erb*B-2 gene in 21MT-2 and 21MT-1 human breast carcinoma cells results in progressively elevated levels of constitutively tyrosine-phosphorylated p185^{erbB-2} and is associated with progressive insulin-like growth factor (IGF) and combined IGF/epidermal growth factor (EGF) independence in culture. In addition, the *neu* differentiation factor/heregulins (HRGs), a family of ligands that activate p185^{erbB-2} through direct binding to *erb*B-3 or *erb*B-4, are potent mitogens for various nonneoplastic mammary epithelial cells and carcinoma cell lines in the absence of both IGF and EGF in culture. We have investigated the ability of ligand induction with HRGs or the constitutive activation of p185^{erbB-2} in the 21MT breast carcinoma cells to induce the recruitment of phosphatidylinositol 3-kinase (PI3K) by p185^{erbB-2} and *erb*B-3. HRG was found to potently induce the recruitment of the M_r 85,000 regulatory subunit of PI3K by phosphotyrosine proteins in both nonneoplastic H16N-2 mammary epithelial cells (which express normal c-*erb*B-2 levels) and in the 21MT-2 and 21MT-1 cell lines, which were all isolated from a single patient with intraductal and invasive ductal carcinoma of the breast and express c-*erb*B-3 but not c-*erb*B-4 in culture. The activation of PI3K in these cells was also associated with high-level mitogenic responsiveness to HRG, as well as the IGF/EGF-independent proliferation of the 21MT cell lines in culture. The recruitment of PI3K by phosphotyrosine proteins during ligand-induced activation, or that seen constitutively in the 21MT tumor cells, did not involve detectable tyrosine phosphorylation of p85. The HRG-induced recruitment of p85 and the constitutive recruitment of p85 in the 21MT cell lines involved direct association with both p185^{erbB-2} and *erb*B-3, although greater levels were recruited directly by *erb*B-3. Wortmannin, a potent inhibitor of PI3K enzymatic activity, also blocked the autonomous proliferation of the 21MT cells, and this effect was reversible in long-term cultures. These data indicate that PI3K may be an especially important mediator of HRG-induced proliferation in mammary epithelial cells and is involved in the autonomous proliferation of growth factor-independent breast carcinoma cells with c-*erb*B-2 gene amplification.

Introduction

The c-*erb*B-2 (*neu*/HER-2) gene encodes an M_r 185,000 protein tyrosine kinase that is highly homologous to EGFR[3] (*erb*B-1), *erb*B-3, and *erb*B-4 (1–3), which together comprise the type 1 receptor tyrosine kinases (4, 5). c-*erb*B-2 is amplified in 28% of primary human breast carcinomas *in vivo* (6), and another 10% overexpress c-*erb*B-2 without amplification of the gene (7–9). In addition, c-*erb*B-2 gene amplification, concordant with high-level overexpression, is correlated with increased tumor aggressiveness and the poor prognosis of breast cancer patients (6, 7, 10–14). Other related genes, such as the *EGFR* gene, are amplified in some human breast cancers (6). However, amplification of the *EGFR* gene in human breast carcinomas is much less common than that seen for c-*erb*B-2 (*i.e.,* 2% *versus* 28%, respectively). Amplification of c-*erb*B-3 or c-*erb*B-4 was not seen in various studies (2, 3). Therefore, we are particularly interested in the specific role of c-*erb*B-2 gene amplification in inducing the oncogenic conversion of human breast cancer cells.

Our laboratory has focused on studying mechanisms of specific growth factor independence in rat and human mammary carcinoma cell lines. We have shown previously that the growth factor-independent proliferation of mammary carcinoma cells in culture is strictly associated with their malig-

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[2] To whom requests for reprints should be addressed, at Department of Radiation Oncology, University of Michigan Medical School, 1331 East Ann Street, Ann Arbor, MI 48109-0582. Phone: (313) 936-7380; Fax: (313) 763-1581.

[3] The abbreviations used are: EGFR, epidermal growth factor receptor; PI, phosphatidylinositol; PI3K, PI 3-kinase; HRG, *neu* differentiation factor/heregulin; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor.

nant potential when serially transplanted *in vivo* (15). Normal mammary epithelial cells require both IGF-I (or supraphysiological levels of insulin) and EGF to proliferate under serum-free conditions in culture (16–18). The synergistic requirement for both IGF and EGF for proliferation of normal mammary epithelial cells suggests that the attainment of autonomous growth in mammary carcinoma cells involves genetic changes that subvert requirements for both IGFs and EGF. We have also recently shown that the 21MT-2 and 21MT-1 breast carcinoma cells, which overexpress progressively elevated levels of c-*erb*B-2, exhibit IGF independence at moderate levels of c-*erb*B-2 overexpression in the 21MT-2 cells and exhibit combined IGF/EGF independence at the highest level of c-*erb*B-2 overexpression in the 21MT-1 cells (79). Furthermore, the HRGs (19, 20), a family of ligands that activate p185^{erbB-2} through direct binding to either *erb*B-3 or *erb*B-4 (21–24), can substitute for IGF and EGF in stimulating the proliferation of nonneoplastic human mammary epithelial cells that coexpress c-*erb*B-2 and c-*erb*B-3 (25). Thus, stimulation with HRG mimics the combined actions of both IGF and EGF in mammary epithelial cells in culture. This also suggests that the combined actions of p185^{erbB-2} and *erb*B-3 mediate the activation of signal transduction mechanisms that substitute for both IGF and EGF during the autonomous proliferation of human breast carcinoma cells with c-*erb*B-2 gene amplification.

The PI3K signal transduction pathway is highly implicated in growth factor-induced mitogenesis through its association with various nonreceptor and receptor tyrosine kinases (reviewed in Ref. 26), including the PDGF (27), colony-stimulating factor 1 (28), interleukin 2 (29), insulin (30), IGF-I (31) and EGF (32) receptors. PI3K was first identified as a component of a complex containing middle T antigen and pp60$^{c\text{-}src}$ in cells transformed by polyoma virus, and its activity was shown to be required for transformation by middle T antigen (33–36). Molecular cloning later showed PI3K to be composed of an M_r 85,000 regulatory subunit (37–39) and a M_r 110,000 subunit (40) that directly mediates its enzymatic function. p85 contains two SH-2 domains that facilitate the binding of PI3K to specific phosphotyrosine residues on both receptor and nonreceptor tyrosine kinases (41–43). Activation of PI3K involves localization of the enzyme to the plasma membrane, where it phosphorylates PI 4-P and PI 4,5-P_2 *in vivo* to generate PI 3,4-P_2 and PI-3,4,5-P_3, respectively (26, 44). PI3K activation may also be directly associated with serine kinase activity (45, 46), and induces the activation of p70^{s6k} (47) and protein kinase Cζ (48). PI3K is now thought to be an especially critical protein for mitogen-dependent signal transduction in that its activity is required for mitogenic responses mediated by various receptor tyrosine kinases, including PDGF (43, 49), colony-stimulating factor 1 (50), insulin (51), and EGF (52) receptors.

To study the signal transduction mechanisms involved in mammary cell transformation in carcinoma cells with c-*erb*B-2 gene amplification, we used a series of nonneoplastic and neoplastic human mammary epithelial cell lines originally isolated from a single patient with intraductal and infiltrating ductal carcinoma of the breast (53–55). These cell lines offer an ideal model system to study the influence of c-*erb*B-2 gene overexpression on growth factor requirements and signal transduction in cell lines that form a progressive series and can be grown under completely defined serum-free conditions in culture. In this study, we have investigated the mitogenic properties and activation of PI3K by HRG. We found that HRG-β is an especially potent inducer of p85 recruitment by both p185^{erbB-2} and *erb*B-3. Constitutive association of p85 with both p185^{erbB-2} and *erb*B-3 was also seen in the c-*erb*B-2-overexpressing 21MT mammary carcinoma cell lines in the absence of exogenous growth factors in culture. Finally, wortmannin, a potent inhibitor of PI3K activity, prevented the autonomous proliferation of the 21MT cells. These data strongly implicate PI3K-mediated signal transduction in HRG-induced mitogenesis, as well as in the autonomous growth of human mammary carcinoma cells with c-*erb*B-2 gene amplification.

Results

Progressive Overexpression and Constitutive Activation of p185^{erbB-2} in 21MT Human Breast Carcinoma Cell Lines. H16N-2, 21MT-2, and 21MT-1 cells were originally isolated from a patient with intraductal and invasive ductal carcinoma of the breast (53–55). The nontransformed H16N-2 cells were isolated from normal tissue (55) and express a low but detectable level of p185^{erbB-2}. The 21MT-2 and 21MT-1 metastatic tumor cell lines isolated from a pleural effusion (52, 53) show equivalent high-level c-*erb*B-2 gene amplification (Fig. 1*A, Lanes 2* and *3*), but 21MT-1 cells express higher levels of c-*erb*B-2 mRNA (Fig. 1*B, Lane 3*). Reprobing of the Southern and Northern blots with a probe complementary to the constitutively expressed *36B4* gene confirmed equivalent loading in these blots (data not shown). Progressively elevated expression of p185^{erbB-2} protein was also measured in Western blots (Fig. 1*C, Lanes 2* and *3*). Furthermore, antiphosphotyrosine immunoprecipitations blotted with anti-erbB-2 antibody showed a direct concordance of c-*erb*B-2 overexpression with constitutive p185^{erbB-2} activation in 21MT cells cultured in the absence of exogenous growth factors (Fig. 1*D, Lanes 2* and *3*). Comparison of the blots shown in Fig. 1, *C* and *D* (which were probed simultaneously with the same anti-erbB-2 antibody) indicates that the proportion of tyrosine-phosphorylated p185^{erbB-2} in the 21MT cell lines is approximately 2–5% of the protein present (*i.e.,* 100 μg of protein was used in Fig. 1*C* and 2 mg of protein was immunoprecipitated in Fig. 1*D*). However, this low stoichiometry still results in easily detectable levels of tyrosine-phosphorlyated p185^{erbB-2}, due to the extremely high levels of p185^{erbB-2} in the 21MT cell lines. Therefore, the H16N-2, 21MT-2, and 21MT-1 cell lines provide a distinct gradient of progressively increasing c-*erb*B-2 gene expression that results in significantly elevated levels of constitutive p185^{erbB-2} tyrosine kinase activity.

We have recently shown that this progressive increase in c-*erb*B-2 gene expression in the H16N-2/21MT cell series is associated with IGF independence in the 21MT-2 cells and combined IGF/EGF independence in the 21MT-1 cells in serum-free culture (79). Therefore, we have sought to further define the changes in the signal transduction events that underlie the growth factor independence that is involved in

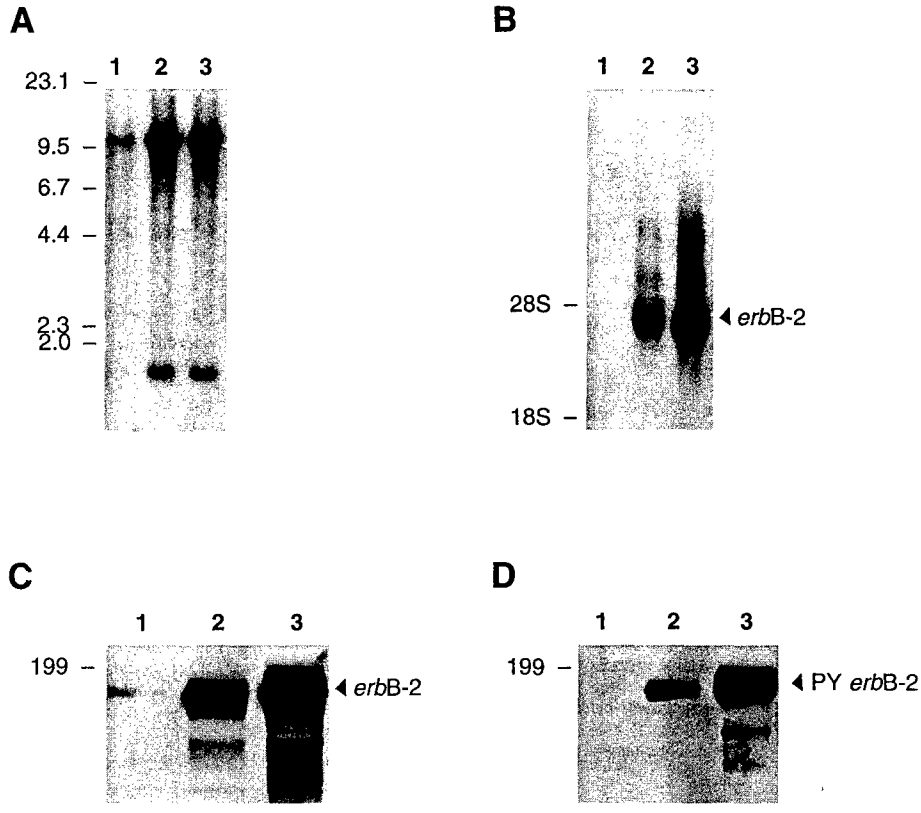

Fig. 1. c-*erb*B-2 gene amplification, overexpression, and constitutive activation of $p185^{erbB-2}$ in 21MT human mammary carcinoma cell lines. H16N-2 (*Lane 1*), 21MT-2 (*Lane 2*), and 21MT-1 (*Lane 3*) cells were used for Southern blot analysis to determine c-*erb*B-2 gene copy number (*A*), Northern blot analysis to determine c-*erb*B-2 mRNA levels (*B*), Western blot analysis using 100 μg lysate protein to determine $p185^{erbB-2}$ levels (*C*), and Western blot analysis of 2 mg lysate protein immunoprecipitated with antiphosphotyrosine antibody to determine the level of tyrosine-phosphorylated $p185^{erbB-2}$ (*D*). For these experiments, the cells were changed to serum-free medium devoid of exogenous growth factors for 48 h prior to protein extractions.

the transformation of these human breast carcinoma cell lines.

HRG Is a Potent IGF/EGF-like Mitogen for Mammary Epithelial Cells in Culture. We previously found that HRG stimulated the proliferation of the nonneoplastic MCF-10A human mammary epithelial cell line in the absence of either IGF or EGF in culture (25). This indicated that HRG acts both as an IGF- and an EGF-like mitogen in mammary epithelial cells that express both $p185^{erbB-2}$ and *erb*B-3 (25). Like the MCF-10A cells, H16N-2, 21MT-2, and 21MT-1 cells all express detectable levels of c-*erb*B-3 but not c-*erb*B-4 in culture (79). In that $p185^{erbB-2}$/erbB-3 heterodimers are known to form high-affinity binding sites for HRG (22), we were also interested in the mitogenic potency of HRG in the H16N-2, 21MT-2, and 21MT-1 cells in culture. HRG stimulated the proliferation of H16N-2, 21MT-2, and 21MT-1 cells in the complete absence of any IGF or EGF in serum-free culture (Fig. 2), indicating that HRG is a very potent IGF/EGF-like mitogen for these cell lines in culture. While the 21MT-1 cells show stimulation of proliferation by HRG, these cells also show extensive proliferation in the absence of both IGF and EGF as well (Fig. 2), which is indicative of their high-level IGF/EGF independence for growth in culture.

PI3K Recruitment by Tyrosine-phosphorylated Proteins Is Potently Stimulated by HRG and Is Constitutively Elevated in Breast Carcinoma Cells with c-*erb*B-2 Gene Amplification. We investigated the role of PI3K activation in the H16N-2, 21MT-2, and 21MT-1 cells by Western blot analysis of p85 levels in cell lysates immunoprecipitated with

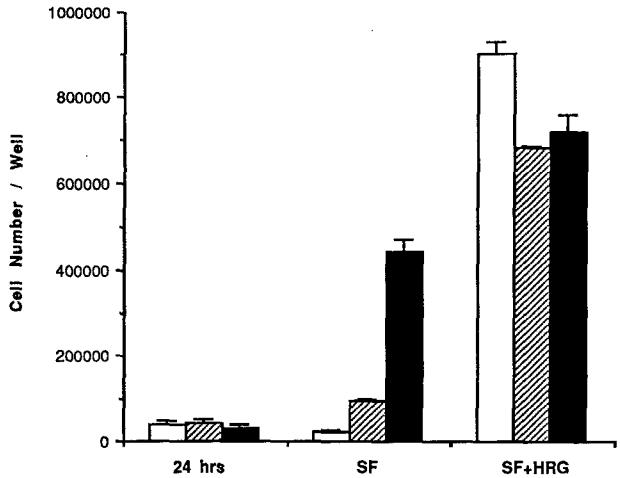

Fig. 2. HRG induces potent mitogenic responses in human mammary epithelial cells in serum-free medium in the absence of both insulin and EGF. H16N-2 (□), 21MT-2 (▨), and 21MT-1 (■) cells were cultured without or with 10 ng/ml HRG-β in serum-free (*SF*) medium without the addition of other growth factors (*i.e.*, IGFs or EGF). Cell counts were taken after 24 h to determine the plating efficiencies and after an additional 9 days by Coulter counting. All conditions were done in triplicate; *bars*, SD.

antiphosphotyrosine antibody (Fig. 3*A*). Stimulation of all three cell lines with HRG induced a very high level of p85 association with antiphosphotyrosine immunoprecipitates (Fig. 3*A*, *Lanes 2, 4,* and *6*). While H16N-2 cells showed no

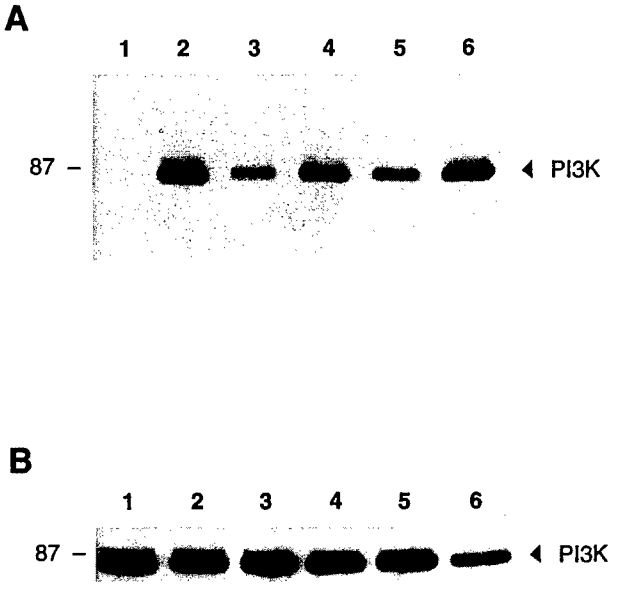

Fig. 3. PI3K p85 recruitment by phosphotyrosine proteins is induced by HRG and is seen constitutively in the 21MT cell lines. *A,* anti-p85 Western blotting of antiphosphotyrosine immunoprecipitates from H16N-2 (*Lanes 1* and *2*), 21MT-2 (*Lanes 3* and *4*), and 21MT-1 (*Lanes 5* and *6*) cells in serum-free medium without growth factors (*Lanes 1, 3,* and *5*) or stimulated with 10 ng/ml HRG-β for 10 min (*Lanes 2, 4,* and *6*). *B,* anti-p85 Western blotting of total cell lysates from the same samples.

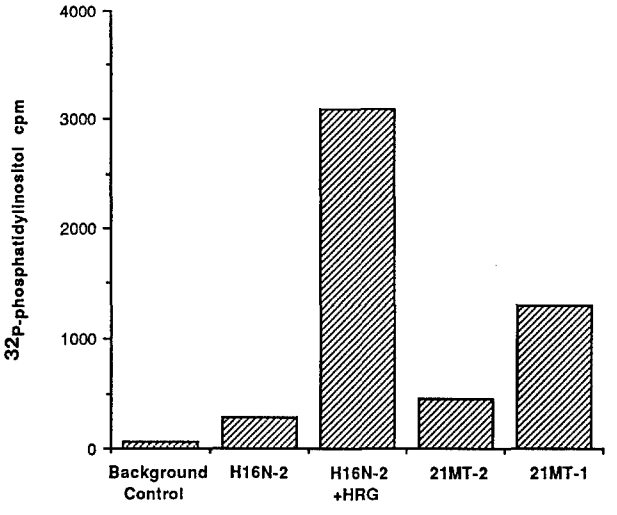

Fig. 4. PI3K enzymatic activity is stimulated by HRG and is constitutively elevated in the 21MT cell lines. Quantification of ^{32}P incorporated into phosphatidylinositol in an assay which measures PI3K activity in antiphosphotyrosine immunoprecipitates from H16N-2, 21MT-2, and 21MT-1 cells in serum-free medium without growth factors. H16N-2 cells were also stimulated with 10 ng/ml HRG-β for 10 min. The mean average of two separate experiments is shown.

detectable level of p85 in antiphosphotyrosine immunoprecipitates in the absence of HRG, both 21MT cell lines showed moderate levels of p85 in antiphosphotyrosine immunoprecipitations in the absence of exogenous growth factors, and this constitutive association of p85 was greater in the 21MT-1 cells (Fig. 3*A, Lane 5*). This indicates that, in addition to being potently induced by HRG, p85 is constitutively recruited by tyrosine-phosphorylated proteins in the 21MT cell lines, and the recruitment of p85 is directly related to the level of c-*erb*B-2 overexpression and constitutive activation of p185^{erbB-2} in these cells (Fig. 1). Western blot analysis of p85 protein levels in total cell lysates collected from the same samples before immunoprecipitation showed that the levels of p85 are comparable in all of the samples (Fig. 3*B*). We also found that the β isoforms of HRG are more potent in inducing p85 recruitment than are the α isoforms (data not shown). This is also consistent with the greater mitogenic potency of the β isoforms in these cell lines (data not shown) and in the MCF-10A cells (25). Therefore, HRG-β was used for ligand induction in these studies.

To determine if the p85 present in antiphosphotyrosine immunoprecipitates was associated with enzymatically active PI3K, assays measuring ^{32}P incorporation into 3′-phosphorylated phosphatidylinositol were performed. As shown in Fig. 4, HRG induced PI3K enzymatic activity in the H16N-2 cells. Moderate levels of PI3K enzymatic activity were also seen constitutively in the 21MT cell lines, with higher levels measured in the 21MT-1 cells.

p85 Is Not Detectably Tyrosine-phosphorylated during Its Activation by HRG or Constitutively in Breast Carcinoma Cells with c-*erb*B-2 Gene Amplification. The antiphosphotyrosine immunoprecipitation/anti-p85 Western blot assay described above detects the proportion of p85 that is associated with tyrosine-phosphorylated proteins and may be due to the phosphorylation of p85 itself. To determine if p85 was being pulled down directly in antiphosphotyrosine immunoprecipitates (*i.e.,* through its own tyrosine phosphorylation) and/or through its association with other tyrosine-phosphorylated proteins, we performed the reciprocal immunoprecipitation/Western blot experiments in which anti-p85 immunoprecipitation was followed by antiphosphotyrosine Western blotting (Fig. 5*A*). No detectable level of tyrosine-phosphorylated p85 was observed after HRG stimulation or constitutively in the 21MT cells. However, M_r 185,000 tyrosine-phosphorylated proteins were detected in anti-p85 immunoprecipitates from cells stimulated with HRG (Fig. 5*A, Lanes 2, 4,* and *6*) and were seen at moderate constitutive levels in the 21MT cell lines (Fig. 5*A, Lanes 3* and *5*). Reprobing of the blots with anti-p85 antibodies confirmed that p85 was effectively immunoprecipitated in all of the samples (Fig. 5*B*). Therefore, p85 is not detectably tyrosine-phosphorylated in these cells during its activation, and the detection of p85 in antiphosphotyrosine immunoprecipitates must be due to its recruitment by other tyrosine-phosphorylated proteins within the cell.

p85 Recruitment by Both p185^{erbB-2} and *erb*B-3 Is Potently Stimulated by HRG and Is Constitutively Elevated in Breast Carcinoma Cells with c-*erb*B-2 Gene Amplification. To determine the relative contribution of the receptors that are directly involved in p85 recruitment induced by HRG or that is seen constitutively in the 21MT cells, we performed Western blot analysis of p85 directly associated with anti-EGFR, anti-*erb*B-2, and anti-*erb*B-3 immunoprecipitates (Fig. 6). HRG induced high-level association of p85 with both p185^{erbB-2} and *erb*B-3 in H16N-2 cells (Fig. 6, *B* and *C, Lane 2*). HRG did not stimulate the recruitment of p85 by EGFR

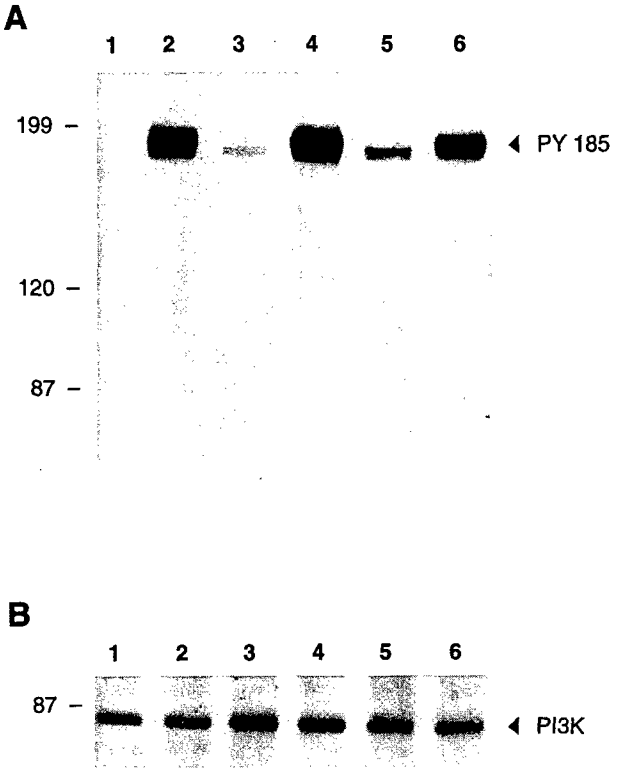

Fig. 5. The p85 subunit of PI3K is not detectably tyrosine-phosphorylated during HRG stimulation or constitutively in the 21MT cell lines. *A*, antiphosphotyrosine Western blotting of anti-p85 immunoprecipitates from H16N-2 (*Lanes 1* and *2*), 21MT-2 (*Lanes 3* and *4*), and 21MT-1 (*Lanes 5* and *6*) cells in serum-free medium without growth factors (*Lanes 1, 3,* and *5*) or stimulated with 10 ng/ml HRG-β for 10 min (*Lanes 2, 4,* and *6*). *B*, the blot shown in *A* was reblotted with anti-p85 antibody.

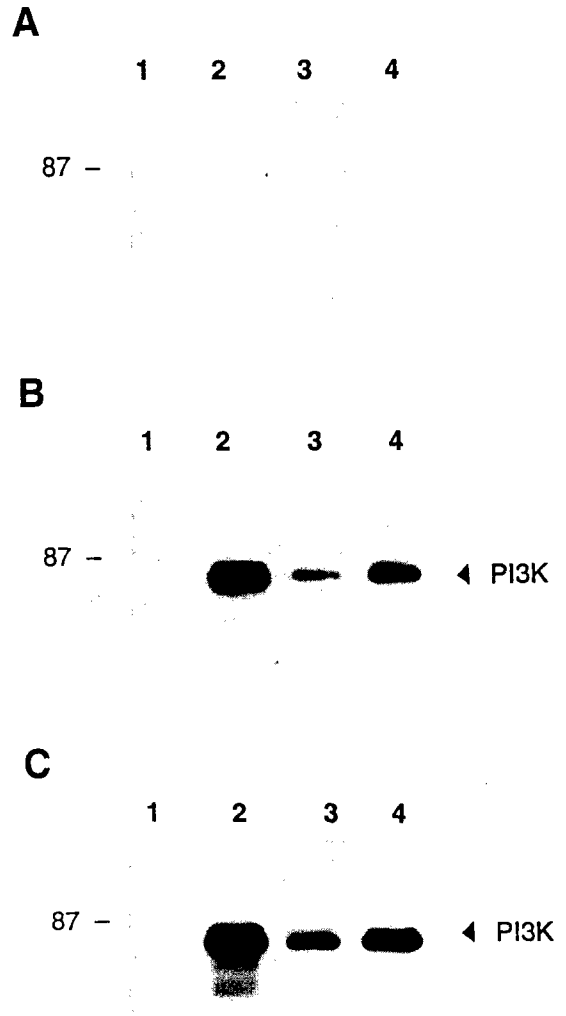

Fig. 6. p85 recruitment by p185^{erbB-2} and *erb*B-3 is stimulated by HRG and is seen constitutively in the 21MT cell lines. Anti-p85 Western blotting of anti-EGFR (*A*), anti-*erb*B-2 (*B*), and anti-*erb*B-3 (*C*) immunoprecipitates from H16N-2 (*Lanes 1* and *2*), 21MT-2 (*Lane 3*), and 21MT-1 (*Lane 4*) cells in serum-free medium without growth factors (*Lanes 1, 3,* and *4*) or for H16N-2 cells stimulated with 10 ng/ml HRG-β for 10 min (*Lane 2*).

(Fig. 6*A*, *Lane 2*). Constitutively elevated levels of p85 were also associated with p185^{erbB-2} and *erb*B-3 in the 21MT-2 and 21MT-1 cell lines in the absence of HRG (Fig. 6, *B* and *C*, *Lanes 3* and *4*) and was greater in the 21MT-1 cells (Fig. 6, *B* and *C*, *Lane 4*). Both the HRG-induced recruitment of p85 and the constitutive recruitment of p85 in the 21MT cell lines showed greater levels directly associated with *erb*B-3. No detectable level of p85 was detected in anti-EGFR immunoprecipitates from the 21MT cells (Fig. 6*A*, *Lanes 3* and *4*), indicating that EGFR does not directly participate in the constitutive recruitment of p85 that is seen in the 21MT cell lines. Reprobing of the blots with anti-EGFR antibody also confirmed that EGFR was being effectively immunoprecipitated in these experiments (data not shown). Also, receptor heterodimers for p185^{erbB-2} and *erb*B-3 were not detected in these samples that were immunoprecipitated without prior cross-linking (data not shown), indicating that noncross-linked receptor dimers are not stable under these immunoprecipitation conditions. Therefore, we conclude that p185^{erbB-2} and *erb*B-3 both directly participate in the recruitment of p85 that is stimulated by HRG or seen constitutively in the 21MT cell lines.

Wortmannin Reversibly Inhibits the Autonomous Proliferation of Mammary Carcinoma Cells with c-*erb*B-2 Gene Amplification. Wortmannin is a potent inhibitor of PI3K enzymatic activity, and it is thought to be specific for PI3K inactivation at submicromolar concentrations (56, 57). Blockade of PI3K activity, either with antibodies or wortmannin, was shown previously to inhibit the growth factor-induced DNA synthesis of various cell types in culture (51, 52). We also found that wortmannin inhibited the growth of the H16N-2 cells when stimulated by insulin and EGF or with HRG (data not shown). We were interested in determining if inactivation of PI3K in the 21MT-1 cells would also effectively inhibit thier autonomous proliferation in culture. Therefore, we added wortmannin to proliferating cultures of 21MT-1 cells grown in serum-free medium devoid of exogenous growth factors. Wortmannin potently inhibited the proliferation of these cells at concentrations specific for PI3K (Fig. 7). Furthermore, the wortmannin inhibition of cell proliferation was reversible in time course experiments in which the wort-

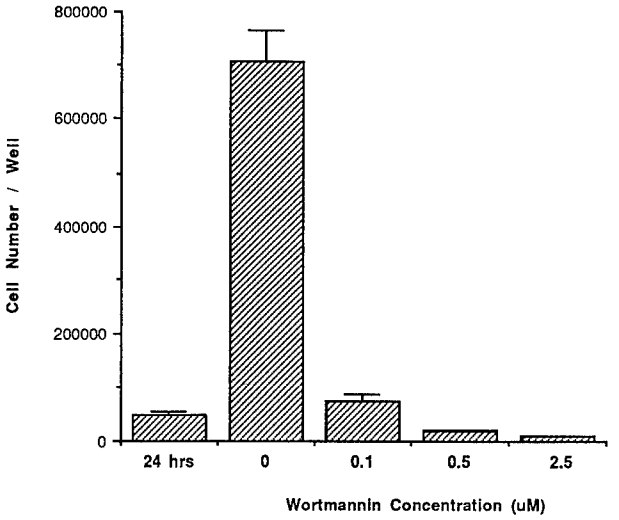

Fig. 7. Wortmannin inhibition of 21MT-1 autonomous cell proliferation in culture. 21MT-1 cells were cultured in serum-free medium without the addition of growth factors for 9 days in the presence of various concentrations of wortmannin. All conditions were done in triplicate; *bars,* SD.

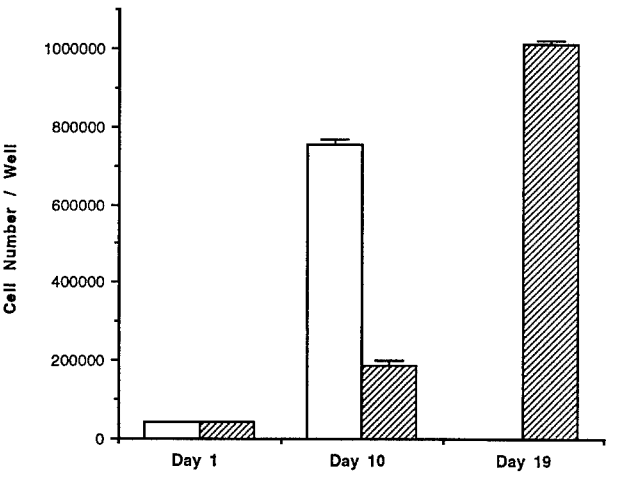

Fig. 8. Wortmannin inhibition of 21MT-1 autonomous cell proliferation is reversible. 21MT-1 cells were cultured in serum-free medium without the addition of growth factors for 9 days in the absence (□) or presence (▨) of 100 nM wortmannin. Wortmannin was then removed, and the cells previously exposed to the drug were cultured another 9 days without the addition of wortmannin. All conditions were done in triplicate; *bars,* SD.

mannin was removed after 10 days in culture (Fig. 8). This reversibility effectively ruled out the possibility that the growth inhibition seen at these concentrations may be due to a general toxicity of the drug. These results suggest that the activation of PI3K is required for the autonomous proliferation of tumor cells that constitutively activate PI3K.

Discussion

We investigated the activation of PI3K that is induced by HRG and that is seen constitutively in human breast carcinoma cell lines with c-*erb*B-2 gene amplification. The gradient of progressively increasing c-*erb*B-2 gene expression in the H16N-2, 21MT-2, and 21MT-1 cells offer a unique opportunity to study the events that underlie the progressive transformation of comparable mammary epithelial cell lines under precisely defined, serum-free conditions in culture. In addition, we are particularly interested in the growth factor-independent phenotypes that occur in cells transformed by p185^{erbB-2} and how constitutive p185^{erbB-2} tyrosine kinase activity may induce various signal transduction pathways normally requiring the combination of both IGF and EGF for activation. We have now shown that p85 recruitment by both p185^{erbB-2} and *erb*B-3 is potently induced by HRG and is constitutively elevated in the 21MT-2 and 21MT-1 cells in the absence of exogenous growth factors in culture. These results indicate that p185^{erbB-2}/*erb*B-3 interactions induce high-level PI3K activation in response to HRG and constitutively activate PI3K in breast carcinoma cells with c-*erb*B-2 gene amplification.

The constitutive activation of p185^{erbB-2} that is routinely seen in mammary carcinomas with c-*erb*B-2 amplification (14) is generally thought to be due to the high-level overexpression of the wild-type receptor, because the transmembrane-mutated form of c-*erb*B-2 has never been detected in human breast carcinoma cells (58). Furthermore, the high-level overexpression of wild-type c-*erb*B-2 causes constitutive activation of p185^{erbB-2} tyrosine kinase function in various cell lines in culture (59–62), including human mammary epithelial cells (62). Transfection of EGFR/c-*erb*B-2 chimeric receptors into cells also results in the constitutive tyrosine kinase activity of the cytoplasmic region of p185^{erbB-2} in the absence of EGF, indicating that the tyrosine kinase domain of p185^{erbB-2} exhibits substantial ligand-independent constitutive activation when sufficiently overexpressed in cells in culture (63, 64). This is consistent with our data, which show that progressively elevated levels of c-*erb*B-2 gene expression in the 21MT-2 and 21MT-1 cells results in progressively elevated levels of constitutive p185^{erbB-2} tyrosine kinase activity in serum-free cultures devoid of exogenous growth factors.

We have previously shown that growth factor independence in culture is strictly associated with the tumorigenicity of serially transplanted mammary carcinoma cells *in vivo* (15). Other workers have also shown a correlation of growth factor independence with increased tumor metastasis in other systems (65). Therefore, growth factor independence, as a phenotype, is a particularly good indicator of progressive neoplasia in tumor cells. We previously developed serum-free culture conditions for the growth of human mammary epithelial cells (18) that allowed us to study the specific growth factor requirements for breast carcinoma cells with c-*erb*B-2 gene amplification (79). Normal human mammary epithelial cells require both IGF and EGF to proliferate under serum-free conditions in culture (16–18). Thus, subversion of IGF and EGF requirements may be especially important for the transformation of breast carcinoma cells that proliferate independently of exogenous growth factors. We have recently shown that the progressive overexpression of p185^{erbB-2} in the 21MT-2 and 21MT-1 cell lines is associated with the attainment of progressive IGF-independent and combined IGF/EGF-independent proliferation in culture (79). Studies

using conditioned media and antireceptor antibody blocking experiments showed that the growth factor independence of the 21MT cell lines is not due to the activation of the IGF-I receptor or the EGFR through an autocrine mechanism (79) but is likely due to the direct effects of constitutive p185^{erbB-2} activation in these cells. Furthermore, we reported previously that activation of p185^{erbB-2} by HRG stimulates the growth of the nonneoplastic MCF-10A human mammary epithelial cells in the absence of either IGF or EGF in culture (25). The MCF-10A cell line, like the H16N-2, 21MT-2, and 21MT-1 cell lines, expresses c-*erb*B-2 and c-*erb*B-3 but not c-*erb*B-4 in culture (25, 79). Here we also show HRG-β to be an especially potent mitogen for the H16N-2 and 21MT cell lines in the complete absence of any exogenous IGF or EGF in serum-free culture. Therefore, HRG stimulation of cells that coexpress c-*erb*B-2 and c-*erb*B-3 mimics the IGF-/EGF-independent phenotype seen in the 21MT-1 mammary carcinoma cells that constitutively overexpress the highest level of tyrosine-phosphorylated p185^{erbB-2}.

Our results showed p85 to be associated with M_r 185,000 tyrosine-phosphorylated proteins in cells stimulated by HRG and was seen constitutively at moderate levels in the 21MT cell lines in serum-free medium devoid of exogenous growth factors in culture. However, p85 was not detectably tyrosine-phosphorylated under any of these conditions. This indicates that the presence of p85 in antiphosphotyrosine immunoprecipitates was due to recruitment by tyrosine-phosphorylated proteins but not to the phosphorylation of p85 itself. Although it has been reported that p85 is tyrosine-phosphorylated during activation of PDGF and insulin receptors (66, 67), this was only seen under *in vitro* conditions or when p85 was highly overexpressed in cell lines transfected with p85 expression vectors (32, 66, 67). Many other reports have confirmed that endogenous p85 is not detectably tyrosine-phosphorylated under more normal conditions during activation, including that seen during neutrophil activation (68) and in different cell types stimulated by various growth factors, including PDGF (32), insulin (30), IGF-1 (31), EGF (32), and when EGFR/*erb*B-3 chimeric receptors are stimulated by EGF (69). These results indicate that tyrosine phosphorylation of endogenous p85 may only occur at a very low stoichiometry, or that p85 is an efficient substrate for protein tyrosine phosphatases under normal conditions *in vivo*.

Our data also showed that the recruitment of PI3K by phosphotyrosine proteins stimulated by HRG, or that seen constitutively in the 21MT cells, was due to the direct recruitment of p85 by both p185^{erbB-2} and *erb*B-3. EGFR did not recruit detectable p85 under these conditions, which did not involve stimulation with EGF. This also indicates that, although all of these cell lines express EGFR (54), EGFR does not directly participate in the constitutive recruitment of PI3K in the 21MT cell lines. It was reported previously that EGFR/*erb*B-3 chimeric receptors are more then 10-fold more potent in stimulating PI3K activation than is wild-type EGFR in NIH 3T3 cells transfected with these genes (69), indicating that *erb*B-3 may be an especially potent activator of PI3K. The carboxyl terminal domain of *erb*B-3 contains multiple YXXM consensus sequences that, when tyrosine-phosphorylated, are known to form high-affinity binding sites for the SH-2 domains of p85 (41–43). In contrast, EGFR, p185^{erbB-2}, and *erb*B-4 contain only one such consensus sequence in their intracellular domains (*e.g.*, at positions 952–955 of unprocessed p185^{erbB-2}). Therefore, it has been speculated that, of the type 1 class of receptor tyrosine kinases, *erb*B-3 principally activates PI3K (5). However, it was also reported previously that the transmembrane-mutated rat *neu* gene directly activates PI3K in fibroblast cells transfected with *neu* (70). Therefore, from data published previously, it seemed likely that both p185^{erbB-2} and *erb*B-3 can activate PI3K. This is consistent with our present results, which show that HRG stimulation induces recruitment of p85 directly by both p185^{erbB-2} and *erb*B-3, albeit the level of p85 recruitment by *erb*B-3 was higher than that seen for p185^{erbB-2}. Furthermore, the recruitment of p85 by p185^{erbB-2} and *erb*B-3 was seen constitutively in the 21MT cells, and this constitutive recruitment of p85 also shows higher levels of p85 directly associated with *erb*B-3. Interestingly, it was recently reported that the YXXM site mentioned above, as well as other sites on EGFR and p185^{erbB-2}, are phosphorylated by pp60$^{c\text{-}src}$ during activation in cells in culture (71). Previous reports have also shown pp60$^{c\text{-}src}$ to be recruited by both EGFR and p185^{erbB-2} during activation (72). These combined data indicate that, in addition to the classical autophosphorylation sites on these receptors, certain sites on these receptors are tyrosine-phosphorylated by pp60$^{c\text{-}src}$ as well (71, 72). These data raise the possibility that pp60$^{c\text{-}src}$ may cooperate with both p185^{erbB-2} and *erb*B-3 to constitutively activate PI3K in mammary carcinoma cells with c-*erb*B-2 gene amplification.

It was previously shown that p185^{erbB-2} and *erb*B-3 form heterodimers that comprise the highest affinity binding sites for HRG in cells genetically engineered to coexpress both proteins (22). Other studies showed that *erb*B-3 is constitutively tyrosine-phosphorylated in a subset of mammary carcinoma cell lines also known to highly overexpress c-*erb*B-2 (73), and this was recently shown to involve heterodimer formation between p185^{erbB-2} and *erb*B-3 (74). Recent studies also showed that c-*erb*B-2 and c-*erb*B-3 cooperate to more effectively transform cells when cotransfected into NIH 3T3 cells, and an increased activation of PI3K was associated with these cooperative effects of p185^{erbB-2} and *erb*B-3 (74, 75). Although these reports did not investigate the direct recruitment of PI3K by p185^{erbB-2}, their data also support a model in which human mammary carcinoma cells that overexpress c-*erb*B-2 constitutively activate PI3K through p185^{erbB-2}/*erb*B-3 interactions. Our data now also strongly implicate both p185^{erbB-2} and *erb*B-3 in directly activating PI3K during high-level mitogenic responses to HRG as well as PI3K which is constitutively activated in growth factor-independent mammary carcinoma cells with c-*erb*B-2 gene amplification.

The importance of constitutive PI3K activation by nonreceptor tyrosine kinases in tumor cells transformed by middle T antigen has been well established (34, 35). In addition, the requirement for PI3K activation in the mitogen-induced responses stimulated by various growth factors has been studied extensively (26, 49–52). However, there are no previous reports showing a requirement for PI3K activation in the

autonomous growth of tumor cells with constitutive activation of p185^{erbB-2}. Our present results also showed that wortmannin inhibited the autonomous proliferation of the 21MT cells at concentrations known to specifically inhibit PI3K activity (56, 57). In addition, this growth inhibition was reversible in long-term cultures, indicating that the inhibition was not due to a general toxicity of wortmannin on the cells at these low concentrations. This indicates that PI3K activation may be required for the constitutive growth of these cells. Ultimately, the introduction of antisense vectors or genes that code for dominant negative forms of p185^{erbB-2}, *erb*B-3, or p85 into these cells may offer great potential for specifically blocking HRG-induced responses or constitutive activation of PI3K in breast carcinoma cells with c-*erb*B-2 gene amplification.

In summary, our results show that HRG is a potent mitogen and inducer of PI3K recruitment in human mammary epithelial cells that express both c-*erb*B-2 and c-*erb*B-3. In addition, the progressive IGF and IGF/EGF independence of 21MT-2 and 21MT-1 cells is associated with progressively increasing constitutive activation of p185^{erbB-2} and with recruitment of PI3K by both p185^{erbB-2} and *erb*B-3. Furthermore, inhibitor studies indicate that the constitutive activation of PI3K by c-*erb*B-2-overexpressing breast carcinoma cells may be necessary for their autonomous growth.

Materials and Methods

Cell Culture. The H16N-2, 21MT-2, and 21MT-1 cell lines were provided by the Dana-Farber Cancer Institute (Boston, MA). The MCF-10A cells were derived by Dr. Herbert Soule at the Michigan Cancer Foundation (Detroit, MI). For routine culture, the cell lines were cultured in F-12 growth medium containing 10 mM HEPES, 5 μg/ml gentamicin, 0.5 μg/ml fungizone, 5 mM ethanolamine, 50 ng/ml sodium selenate, 10 mM triiodothyronine, 1 μg/ml hydrocortisone, 5 μg/ml transferrin, 5 μg/ml insulin, 10 ng/ml EGF, 0.1 mg/ml BSA, and 2% FBS. For serum-free growth experiments, the FBS was omitted after 24 h. Cells were cultured at 37°C with 10% carbon dioxide in a humidified incubator, and the medium was changed every other day. For subculture, the cells were rinsed in calcium- and magnesium-free HBSS and then in 0.05% trypsin plus 0.025% EDTA in calcium- and magnesium-free HBSS. After aspiration of the trypsin solution, the cells were incubated at 37°C for 5–15 min, and the released cells were immediately resuspended in growth medium for replating in 60- or 100-mm tissue culture plates. Cells were counted with a hemocytometer or Coulter counter and plated at a density of 10^4 cells/cm^2.

Southern Blot Analysis. Standard techniques were used to isolate genomic DNA from the cell lines in culture (76). For DNA isolation, the different cell lines were grown to confluence in 100-mm plates and were lysed directly in the plates with DNA extraction buffer before extraction with phenol/chloroform. Southern blots were prepared as described previously (76) with some minor modifications. Ethidium bromide (final concentration, 0.5 μg/ml) was added directly to the agarose gel before polymerization. For each sample, 15 μg of DNA were digested with 30 units of restriction enzyme (*Hin*dIII) at 37°C for 2 h. The reaction was stopped by the addition of EDTA (final concentration, 25 mM) and incubation at 65°C for 10 min. The digested DNA was electrophoresed through 0.7% agarose gels in Tris acetate/EDTA buffer for approximately 16 h at 15 V constant voltage. Denaturation in NaOH was performed (76) after the gel was photographed. The acid treatment step with depurination was omitted. The DNA was transferred onto Nytran nylon membrane (Schleicher & Schuell) by capillary action atop a sponge and blotting paper in 10× SSC buffer for approximately 24 h. The blots were then UV cross-linked according to the manufacturer's instructions (Stratagene) and stored wrapped in plastic for later use.

For hybridizations, the blots were prehybridized with mild agitation in a small plastic container with 20 ml hybridization solution (5× SSC, 50% formamide, 5× Denhardt's solution, 50 mM potassium phosphate, and 0.1% SDS and 100 μg/ml denatured salmon sperm DNA) at 42°C for 2–12 h. The human c-*erb*B-2 and *36B4* probes were prepared from low-melting-point, agarose gel-purified inserts by labeling with [^{32}P]dCTP (>3000 Ci/mmol) by random primer extension according to the manufacturer's instructions (Life Technologies, Inc.). Probes with specific activities of 8×10^8–4×10^9 cpm were used in these experiments. Radiolabeled probe (10^7 cpm/ml) was added to the hybridization solution and incubated with mild agitation at 42°C for 24 h. The blots were then washed with moderate agitation twice for 10 min each in 2× SSC plus 0.1% SDS at 20°C, 0.2× SSC plus 0.1% SDS at 42°C, and 0.1× SSC plus 0.1% SDS at 65°C. The washed blots were then used to expose X-ray (Kodak) film with intensifying screens for 2–14 days at −70°C and were developed in D19 developer (Kodak). The blots were stripped for reprobing by washing in 0.01× SSC plus 0.1% SDS at 65°C for 1 h.

Northern Blot Analysis. Total cellular RNA was isolated from subconfluent cell cultures by guanidine/phenol/chloroform extraction as described previously (77). RNA was extracted from cell cultures by lysing the cells in guanidine solution directly in the plates. RNA was resuspended in diethylpyrocarbonate-treated water for spectrophotometric quantification, and 20-μg samples of RNA were electrophoresed through 1% agarose gels containing formaldehyde, as described previously (78) with some minor modifications. Gels were run for approximately 20 h at 15 V constant voltage and were stained with 0.5 μg/ml ethidium bromide in 0.2 M ammonium acetate for 45 min with mild agitation before photographing the gel and transferring the samples to nylon membrane, as described above for the Southern blotting. Hybridizations were carried out exactly as described above for the Southern blots.

Western Blot Analysis and Immunoprecipitations. Subconfluent cell cultures were incubated in serum-free medium without IGF and EGF for 48 h before isolation of cell lysates for Western blots. For ligand induction, cells were incubated in prewarmed, serum-free medium without IGF and EGF containing 10 ng/ml HRG for 10 min prior to lysate preparation. Cells were lysed in immunoprecipitation buffer [150 mM NaCl, 50 mM Tris (pH 7.4), 0.5% NP40, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 mM sodium PP_i, and 5 mM sodium orthovanadate], and the samples were spun at 14,000 × *g* for 15 min. The lysate protein was assayed using the Bradford assay (Bio-Rad). For all nonimmunoprecipitated samples, 100 μg lysate protein were loaded for each sample. For immunoprecipitations, 2 mg lysate protein was immunoprecipitated for each sample with primary antibody or antiphosphotyrosine beads for 2 h with agitation at 20°C. For unconjugated antibodies, 50 μl protein A- or protein G-agarose (Sigma Chemical Co.) was then added and incubated another hour with agitation at 4°C. Thirty μl of antiphosphotyrosine beads were used per sample for antiphosphotyrosine immunoprecipitations. Anti-p85 antiserum was used at a 1:250 dilution, anti-EGFR (Ab-1) monoclonal antibody was used at a 1:50 dilution, anti-c-*neu* (Ab-5) monoclonal antibody was used at a 1:50 dilution, and anti-*erb*B-3 (3C3) monoclonal antibody supernatant was used at a 1:20 dilution. After three washes in immunoprecipitation buffer, the agarose pellets were resuspended in sample buffer and electrophoresed in 7.5% SDS-PAGE gels for approximately 20 h at 15 mA constant current. The samples were then transferred onto Immobilon-P membranes (Millipore) by overnight electrotransfer in standard transfer buffer at 125 mA, followed by 2 h at 325 mA. The blots were equilibrated in TTBS [150 mM NaCl, 50 mM Tris (pH 7.5) plus 0.1% Tween 20], incubated in TTBS containing 3% nonfat dry milk at 20°C for 1 h with mild agitation, and then with primary antibody in TTBS plus 3% milk for 1 h. Anti-erbB-2 (Pab 9.3) antiserum, anti-p85 antiserum, and antiphosphotyrosine monoclonal antibody (PY-20) were all used at a 1:500 dilution for Western blotting. The blots were rinsed in TTBS three times with moderate agitation for 5 min each after antibody binding steps. Biotinylated antirabbit IgG or antimouse IgG secondary antibodies were used at a 1:5000 and 1:1000 dilution, respectively. Enhanced chemiluminescent (Amersham) or Vectastain ABC streptavidin horseradish peroxidase reagents (Vector Laboratories) were used to visualize bands according to the manufacturer's instructions. Anti-p85 (UBI), anti-EGFR (Oncogene), anti-c-*neu* (Oncogene), antiphosphotyrosine (ICN), and antiphosphotyrosine antibody-agarose conjugate (Oncogene) were purchased from commercial sources. The Pab 9.3 anti-*erb*B-2 polyclonal antibody was provided by Dr. Beatrice Langton at Berlex Biosciences (Richmond, CA), and the 3C3 anti-*erb*B-3 monoclonal antibody was kindly supplied by Dr. Atul Tandon at NeoMarkers (Fremont, CA).

PI3K Enzymatic Assay. Antiphosphotyrosine immunoprecipitations were performed as described above with additional washes as described previously (32). Assay for PI3K enzymatic activity used ^{32}P incorporation into phosphatidylinositol (Sigma) and TLC as described previously (32). After exposure of X-ray film to the TLC plates, the spots formed by migration of 3'-phosphorylated phosphatidylinositol were cut out of plastic TLC plates (EM Industries) and counted in scintillation fluid.

Proliferation Assays. For the growth assays, the cell lines were plated in 6-well tissue culture plates at a density of 5×10^4 cells/well (*i.e.,* 5×10^3 cells/cm^2) in growth medium containing all of the factors listed above minus the insulin and EGF. After 24 h, the medium was replaced with serum-free medium without insulin and EGF. Cell counts were taken after 24 h to measure the plating efficiency and at day 10 to measure the proliferation after 9 days in culture. For counting cells, the cell nuclei from triplicate wells for each condition were detergent solubilized and counted in a Coulter counter as described previously (18). The recombinant α and β isoforms of HRG were kindly provided by Amgen Inc. For the experiments using wortmannin (Sigma), 0.1% DMSO (for controls) or wortmannin dissolved in DMSO was added fresh daily to the cell cultures.

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Insulin-Like Growth Factor and Epidermal Growth Factor Independence in Human Mammary Carcinoma Cells With c-*erb*B-2 Gene Amplification and Progressively Elevated Levels of Tyrosine-Phosphorylated p185^{erbB-2}

Tracy G. Ram, Cheryl A. Dilts, Michele L. Dziubinski, Lori J. Pierce, and Stephen P. Ethier[1]

Department of Radiation Oncology, The University of Michigan Medical School, Ann Arbor, Michigan

Growth factor–independent proliferation is an essential aspect of the transformation process. To study the influence of c-*erb*B-2 overexpression on the autonomous growth of human mammary cancer cells, we used a series of non-neoplastic and neoplastic human mammary epithelial cell lines isolated from a patient with intraductal and invasive ductal carcinoma of the breast. The non-neoplastic cell line, H16N-2, which expresses a normal level (single gene copy) of c-*erb*B-2, was used for comparison with the neoplastic cell lines. Both the metastatic tumor cell lines, 21MT-1 and 21MT-2, showed equivalent amplification of the c-*erb*B-2 gene; however, 21MT-1 cells showed a higher level of c-*erb*B-2 overexpression. Therefore, the H16N-2, 21MT-2, and 21MT-1 cell series forms a distinct gradient of progressively increasing c-*erb*B-2 gene expression. Furthermore, the overexpression of c-*erb*B-2 in the 21MT cell lines was concordant with increases in the constitutive tyrosine kinase activity of p185^{erbB-2} measured in the absence of exogenous growth factors in culture. Normal mammary epithelial cells require both insulin-like growth factor (IGF)-1 (or supraphysiological concentrations of insulin) and epidermal growth factor (EGF) to proliferate under serum-free conditions in culture. By contrast, 21MT-2 cells showed a reduced requirement for IGF but still required EGF to proliferate. 21MT-1 cells did not require either insulin or EGF to proliferate. Therefore, the progressive increases in constitutive p185^{erbB-2} tyrosine kinase activity in the 21MT-2 and 21MT-1 cell lines was directly correlated with IGF independence and combined IGF and EGF independence under defined conditions in culture. Experiments using conditioned media and anti–IGF-1 receptor and anti–EGF receptor neutralizing antibodies showed that the growth-factor independence of the tumor cells did not involve detectable IGF- or EGF-like autocrine activity expressed by the 21MT cells. Furthermore, *neu* differentiation factor/heregulin, a ligand that indirectly activates p185^{erbB-2} by direct binding to erbB-3 receptors, potently stimulated the proliferation of the growth factor–dependent H16N-2 cells (which expressed c-*erb*B-2 and c-*erb*B-3 but not c-*erb*B-4) in the absence of both IGF and EGF. Thus, HRG-induced mitogenesis mimicked the autonomous growth seen in the 21MT cells that have the highest level of constitutive p185^{erbB-2} activation. These data support the hypothesis that the constitutive activation of p185^{erbB-2} in human mammary carcinoma cells causes growth-factor independence by directly activating multiple signal-transduction pathways that substitute for both IGF and EGF during proliferation.

Key words: Mammary epithelium, tyrosine kinase receptors, gene amplification, transformation, tumor progression

INTRODUCTION

The c-*erb*B-2 (*neu*/*HER-2*) gene encodes a 185-kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity that is highly homologous to the erbB-1 (epidermal growth factor receptor (EGFR)), erbB-3, and *erb*B-4 receptors [1–3]. Together, these proteins comprise the type I family of single-transmembrane receptor tyrosine kinases [4]. The c-*erb*B-2 gene is amplified in 28% of primary human breast carcinomas in vivo, and this amplification is correlated with increased tumor aggressiveness and poor prognosis for breast cancer patients [5–11]. Additionally, 10% of primary breast carcinomas overexpress c-*erb*B-2 without amplification of the gene [11]. This indicates that more than a third of human breast cancers overexpress c-*erb*B-2 through various genetic or epigenetic mechanisms (or both) [12,13]. Overexpression of c-*erb*B-2 has also been reported in carcinomas of the stomach [14], ovary [11], colon [15], lung [16], pancreas [17], and esophagus [18]. However, it is not yet entirely clear why c-*erb*B-2 is an especially susceptible target for overexpression

[1]Corresponding author: Stephen P. Ethier, Department of Radiation Oncology, The University of Michigan Medical School, 1331 E. Ann St., Ann Arbor, MI 48105-0582.

Abbreviations: IGF, insulin-like growth factor; EGF, epidermal growth factor; HRG, *neu* differentiation factor/heregulin; RT-PCR, reverse transcription–polymerase chain reaction; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; TTBS, tris-buffered saline plus Tween-20; RT, reverse transcription; PCR, polymerase chain reaction; PI3K, phosphatidylinositol-3-kinase; PLCγ, phospholipase Cγ.

that leads to the oncogenic conversion of cancer cells. While other related genes (such as the *EGFR* gene) are sometimes amplified in primary breast carcinomas in vivo [5], this is much less common than c-*erb*B-2 amplification (2% vs 28%, respectively). Therefore, we are interested in the specific properties of the erbB-2 protein that make it an especially potent oncoprotein when overexpressed in human mammary epithelial cells. The level of c-*erb*B-2 overexpression seen in primary breast carcinomas in vivo corresponds directly with the level of tyrosine-phosphorylated $p185^{erbB-2}$ [19], indicating that overexpression of $p185^{erbB-2}$ constitutively activates its tyrosine kinase function. In addition, the protein encoded by the wild-type c-*erb*B-2 gene has been shown to exhibit constitutive tyrosine kinase activity if sufficiently overexpressed in a variety of cell lines in culture [20–24], and a c-*erb*B-2 gene with mutated transmembrane domains (like the rat *neu* gene) has never been detected in human mammary carcinomas [25]. Therefore, the constitutive activation of $p185^{erbB-2}$ seen in human mammary carcinoma cells is thought to be due to the high level of overexpression of the wild-type protein.

Our laboratory is determining how cancer cells develop growth autonomy by studying alterations in specific growth-factor requirements during tumor progression. We have previously shown that the attainment of insulin-like growth factor (IGF)-independent or epidermal growth factor (EGF)-independent (or both) proliferation of mammary carcinoma cells in culture is strictly associated with their tumorigenic potential when serially transplanted in vivo [26]. Both non-autocrine [27] and autocrine [28] mechanisms are involved in these phenomena, depending on the particular tumor and stage of the disease. Normal human mammary epithelial cells strictly require both IGF-1 (or supraphysiological levels of insulin) and EGF to proliferate under serum-free conditions in culture [29–31]. This synergistic requirement for both IGF and EGF suggests that multiple signal–transduction pathways must be stimulated by different receptors for normal mammary epithelial cells to proliferate. Therefore, the attainment of autonomous growth potential in mammary carcinoma cells may involve genetic changes that affect signal-transduction pathways normally activated by IGFs and EGF-related growth factors. By using well-defined serum-free culture conditions, we attempted to integrate what is known about the growth-factor requirements of normal human mammary epithelial cells with how the requirements are altered in c-*erb*B-2–overexpressing human mammary carcinoma cells.

In the study presented here, we used a series of non-neoplastic (H16N-2) and neoplastic (21MT-1 and 21MT-2) cell lines originally isolated from a patient with intraductal and infiltrating ductal carcinoma of the breast [32–35]. We quantified the level of c-*erb*B-2 gene amplification and overexpression and $p185^{erbB-2}$ tyrosine kinase activity in these cells and investigated how these alterations in c-*erb*B-2 gene expression are involved in the growth factor–independent proliferation of the 21MT cell lines in culture. We found that all the cell lines expressed c-*erb*B-3 but not c-*erb*B-4, indicating that *erb*B-3 may be involved in the growth-factor independence of 21MT cells. Furthermore, we investigated the influence of ligand activation of $p185^{erbB-2}$ and erbB-3 by neu differentiation factor/heregulin (HRG) on the growth-factor requirements of the growth factor–dependent mammary epithelial cells. The HRGs are a family of ligands that are known to activate $p185^{erbB-2}$ indirectly by binding directly to either erbB-3 or erbB-4 [36–39]. Our results indicate that either the ligand-induced or constitutive activation of $p185^{erbB-2}$ in human mammary epithelial cells induces cell proliferation in a manner similar to that usually requiring both IGF and EGF.

MATERIALS AND METHODS

Cell Culture

The H16N-2, 21MT-2, and 21MT-1 cell lines were provided by Dr. Vimla Band (Dana-Farber Cancer Institute, Boston, MA). The MCF-10A cell line was developed by Dr. Herbert Soule (Michigan Cancer Foundation, Detroit, MI). SK-BR-3 and T47D cells were purchased from the American Type Culture Collection (Rockville, MD). MCF-10A, H16N-2, 21MT-1, and 21MT-2 cells were cultured in F-12 medium containing 10 mM HEPES, 5 μg/mL gentamycin, 0.5 μg/mL fungizone, 5 mM ethanolamine, 50 ng/mL sodium selenate, 10 mM triiodothyronine, 1 μg/mL hydrocortisone, 5 μg/mL transferrin, 5 μg/mL insulin, 10 ng/mL EGF, 0.1 mg/mL bovine serum albumin, and 2% fetal bovine serum. SK-BR-3 and T47D cells were cultured in Dulbecco's modified Eagle's medium containing 5 μg/mL gentamycin, 0.5 μg/mL fungizone, and 10% fetal bovine serum. All cells were cultured at 37°C with 10% carbon dioxide in a humidified incubator, and the medium was changed every other day. To establish subcultures, the cells were rinsed in calcium- and magnesium-free Hanks' balanced salt solution then in 0.05% trypsin plus 0.025% EDTA in calcium- and magnesium-free Hanks' balanced salt solution. After aspiration of the trypsin solution, the cells were incubated at 37°C for 5–15 min, and the released cells were immediately resuspended in growth medium for replating in 60- or 100-mm tissue-culture plates. For routine culture, cells were counted with a hemocytomer or Coulter counter (Coulter Electronics, Hialeah, FL) and plated at a density of 10^4 cells/cm^2.

Southern-Blot Analysis

Standard techniques were used to isolate genomic DNA from the cell lines in culture [40]. The different

cell lines were grown to confluence in 100-mm plates for DNA isolation, and the cells were lysed directly in the plates with DNA extraction buffer before extraction with phenol and chloroform. Southern blots were prepared as previously described [40], with some minor modifications. Ethidium bromide (0.5 μg/mL final concentration) was added directly to the agarose gel before it solidified. Fifteen micrograms of DNA was digested with 30 U of restriction enzyme at 37°C for 2 h. The reactions were stopped by addition of EDTA (25 mM final concentration) and incubated at 65°C for 10 min. The digested DNA was electrophoresed through 0.7% agarose gels in Tris-acetate-EDTA buffer for approximately 16 h at 15 V constant voltage. Denaturation with NaOH was performed [40] after the gel was photographed. The acid treatment with depurination was omitted. The DNA was transferred onto Nytran nylon membrane (Schleicher & Schuell Inc., Keene, NH) by capillary action atop a sponge and blotting paper in 10× standard saline citrate (SSC) for approximately 24 h. The blots were then cross-linked with ultraviolet light with a Stratalinker (Stratagene, La Jolla, CA) by following the manufacturer's instructions and were stored wrapped in plastic for later use.

For hybridizations, the blots were prehybridized with mild agitation in a small plastic container with 20 mL of hybridization solution (5× SSC, 50% formamide, 5× Denhardt's solution, 50 mM potassium phosphate, 0.1% sodium dodecyl sulfate (SDS), and 100 μg/mL denatured salmon-sperm DNA) at 42°C for 2–12 h. The human c-*erb*B-2 [20] and 36B4 [41] probes were prepared from inserts isolated from low-melting-point agarose gels and labeled with [^{32}P]dCTP (more than 3000 Ci/mmol) by the random-primer extension method according to the manufacturer's instructions (BRL, Gaithersburg, MD). Probes with specific activities of 8×10^8 to 4×10^9 cpm were used for these experiments. Radiolabeled probe (10^7 cpm/mL) was added to the hybridization solution and incubated with mild agitation at 42°C for 24 h. The blots were then washed with moderate agitation twice for 10 min each time in 2× SSC plus 0.1% SDS at 20°C, then in 0.2× SSC plus 0.1% SDS at 42°C, and finally in 0.1× SSC plus 0.1% SDS at 65°C. The washed blots were then exposed to x-ray film (Eastman Kodak Co., Rochester, NY) with intensifying screens for 2–14 d at –70°C, and the film was developed in D19 developer (Eastman Kodak Co.). The blots were stripped for reprobing by washing them in 0.01× SSC plus 0.1% SDS at 65°C for 1 h.

Northern-Blot Analysis

Total cellular RNA was isolated from the cell lines in culture by guanidine-phenol-chloroform extraction as previously described [42]. RNA was extracted from cell cultures by lysing the cells in guanidine solution directly in the plates. The RNA was resuspended in diethylpyrocarbonate-treated water for spectrophotometric quantification, and 20-μg samples of RNA were electrophoresed through 1% agarose gels containing formaldehyde as previously described [43] with some minor modifications. The gels were run for approximately 20 h at 15 V constant voltage and were stained with 0.5 μg/mL ethidium bromide in 0.2 M ammonium acetate for 45 min with mild agitation before photographing the gel and transferring the samples to nylon membrane as described above for Southern blotting. Hybridizations were performed exactly as described above for the Southern blots.

Immunoprecipitation and Western-Blot Analysis

Cell cultures were incubated in serum-free medium overnight without EGF before isolation of membrane proteins for western blotting. Membrane proteins were prepared for western blotting by scraping cell cultures with a rubber policeman in 20 mM HEPES buffer containing 5 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and 1 mM phenylmethylsulfonyl fluoride. The samples were homogenized with a Dounce homogenizer 50 times and centrifuged at 800 × *g* for 10 min. Membrane proteins were then isolated by centrifuging the supernatant at 100 000 × *g* for 30 min, and the pellet was resuspended in Laemlli sample lysis buffer. Membrane protein was measured by using a Lowry assay. For immunoprecipitation, the cells were lysed in immunoprecipitation buffer (150 mM NaCl; 50 mM Tris, pH 7.5; 0.5% NP-40; 5 mM EDTA; 5 mM sodium orthovanidate; 10 mM sodium pyrophosphate; and 2 mM phenylmethylsulfonyl fluoride), centrifuged for 15 min at 14000 × *g*, incubated with 2 μg/mL Ab-5 anti–erbB-2 monoclonal antibody (Oncogene Science Inc., Uniondale, NY) for 2 h with agitation and with protein G-agarose (Sigma Chemical Co., St. Louis, MO) for 1 h before they were washed three times in immunoprecipitation buffer and boiled in electrophoresis sample buffer. Samples were electrophoresed in 7.5% SDS-polyacrylamide gels for approximately 5 h at 60 mA. The samples were transferred onto Imobilon-P membranes (Millipore Corp., Bedford, MA) by electrotransfer in transfer buffer overnight at 125 mA and then 2 h at 325 mA. The blots were equilibrated in Tris-buffered saline plus Tween-20 (TTBS; 150 mM NaCl; 50 mM Tris, pH 7.5; and 0.1% Tween-20), incubated in TTBS containing 3% nonfat dry milk at room temperature for 30 min with mild agitation, and incubated with either Pab 9.3 anti–erbB-2 polyclonal antibody (a gift from Dr. Beatrice Langton, Berlex Biosciences, Richmond, CA) or PY-20 anti-phosphotyrosine monoclonal antibody (ICN, Cleveland, OH) diluted 1:500 in TTBS plus 3% milk at room temperature for 30 min. The blots were rinsed in TTBS three times with moderate agitation for 5 min time each after the antibody-binding steps. Biotinylated anti–rabbit IgG or anti–mouse immunoglobin G secondary antibodies were used diluted 1:1000 dilution.

Vectastain ABC strepavidin HRP reagents were used to visualize bands with diaminobenzidine as a substrate according to the manufacturer's instructions (Vector Laboratories Inc., Burlingame, CA).

Proliferation Assay

For the standard growth assays, the cell lines were plated in six-well tissue-culture plates at a density of 5×10^4 cells/well (i.e., 5×10^3 cells/cm^2) in growth medium containing all of the factors listed above except insulin and EGF. After 24 h, the medium was replaced with serum-free medium containing the other supplements without either insulin or EGF. The cells were counted after 24 h to measure the plating efficiency and after 10 d to measure proliferation during the previous 9 d in culture. For the HGR experiments, the cells were plated at 10^5 cells/well and were counted after 8 d in culture. For cell counting, the cell nuclei from triplicate wells for each condition were solubilized with detergent and counted in a Coulter counter as previously described [44]. For the conditioned media experiments, serum-free medium devoid of either insulin or EGF was collected from confluent cultures of H16N-2, 21MT-2, 21MT-1, and 8-12 RMT cell lines after 48 h of incubation, cell debris was removed by centrifugation at 1500 rpm for 10 min, and the medium was diluted 1:1 with fresh medium before it was added to MCF-10A cell cultures for the growth assay. Anti–IGF-1 receptor and anti-EGFR monoclonal antibodies were purchased from Oncogene Science Inc. (Uniondale, NY) Recombinant HRG-β was provided by Amgen Biologicals (Thousand Oaks, CA).

Polymerase Chain Reaction Amplification of Reverse-Transcribed RNA

RNA was prepared by the RNAzol RNA isolation procedure according to the manufacturer's instructions (Tel-Test, Friendswood, TX). After isopropanol precipitation, the RNA pellet was resuspended in diethylpyrocarbonate-treated water, and aliquots were added to a reverse transcription (RT) reaction and incubated at 41°C for 1 h. For the RT reactions, we used the antisense primers CCCCCGGCACACTCATCAT and AGCAGCCTCCAGCACATTCTC, which are 3′ of the region used for polymerase chain reaction (PCR) amplification of c-*erb*B-3 and c-*erb*B-4, respectively. For PCR amplification, the thermocycling conditions were 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min for a total of 30 cycles. For c-*erb*B-3, the primers GACCGGCGATGCTGAGAACCAA (sense) and GGGCCCAAAGCAGTGACCATTACA (antisense) were used to amplify a 550-bp fragment that corresponds to nt 213–739 of the c-*erb*B-3 cDNA [2]. For c-*erb*B-4, the primers CCTCTCCTTCCTGCGGTCTGT (sense) and AAGTCTGGCAATGATTTTCTGTGG (antisense) were used to amplify a 404-bp fragment that corresponds to nt 252–632 of c-*erb*B-4 cDNA [3]. For both the RT and PCR reactions, the standard buffers supplied with avian myeloma virus reverse transcriptase and Taq polymerase were used according to the manufacturer's instructions (Boehringer Mannheim Corp., Indianapolis, IN). The RT-PCR samples were electrophoresed in 4% Nusieve agarose gels in buffer at 80 V constant voltage for approximately 1–2 h, stained with 0.5 μg/mL ethidium bromide, and photographed.

RESULTS

Cell Lines

For these studies, we used a series of cell lines originally isolated from a patient with intraductal and infiltrating ductal carcinoma of the breast that was estrogen receptor negative and EGFR positive [32–35]. H16N-2 cells are non-neoplastic mammary epithelial cells isolated from the normal mammary epithelial tissue that was immortalized by transfection with human papilloma virus (HPV) 16 and are not tumorigenic in nude mice [35]. The 21MT metastatic mammary carcinoma cell lines 21MT-1 and 21MT-2 were isolated from a pleural effusion collected when metastatic disease developed 1 yr after chemotherapy and are highly tumorigenic in nude mice [33,34]. H16N-2, 21MT-1, and 21MT-2 cells all express EGFR in culture [34]. Restriction fragment length polymorphism analysis previously showed that these cell lines share common genetic polymorphisms [34]. We have also verified that the H16N-2, 21MT-1, and 21MT-2 cell lines were derived from a single individual by PCR–DNA fingerprinting analysis of a hypervariable region of the *BRCA-1* locus (data not shown).

Gene Amplification and Overexpression of Constitutively Active *erb*B-2 in 21MT Cells

We measured the c-*erb*B-2 gene copy numbers in the H16N-2 and 21MT cell lines by Southern blot analysis. To compare the results with a non-neoplastic cell line known to not have c-*erb*B-2 gene amplification, we included the non-neoplastic MCF-10A human mammary epithelial cell line [45,46] in these experiments. Southern-blot analysis showed that H16N-2 cells had a normal c-*erb*B-2 gene copy number, whereas both of the metastatic tumor cell lines (21MT-1 and 21MT-2) had equivalent amplification of the c-*erb*B-2 gene (Figure 1A). To confirm equal loading of the DNA, the blots were reprobed for 36B4 (Figure 1B), a constitutively expressed gene that has not been reported to show gene amplification or diminution.

The levels of c-*erb*B-2 gene expression in the different cell lines were measured by northern-blot analysis of total RNA extracted from the cell lines in culture (Figure 2A). H16N-2 cells showed c-*erb*B-2 mRNA levels comparable to those of MCF-10A cells, in which c-*erb*B-2 mRNA was only seen faintly after longer exposures of the blots (data not shown). Both 21MT cell lines showed high levels of overexpression

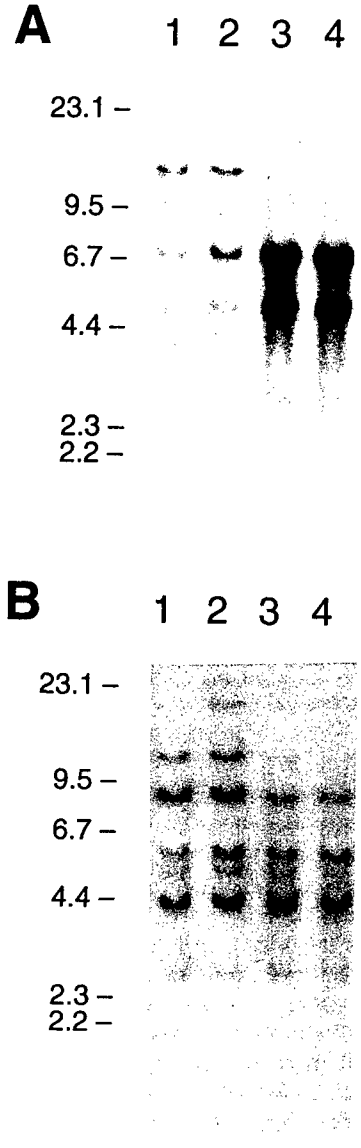

Figure 1. Amplification of the c-*erb*B-2 gene in 21MT cells. Southern-blot analysis of c-*erb*B-2 gene copy number in MCF-10A cells (lane 1), H16N-2 cells (lane 2), 21MT-2 cells (lane 3), and 21MT-1 cells (lane 4). Fifteen micrograms of genomic DNA from each cell line was digested with EcoRI and used to prepare Southern blots hybridized with ^{32}P-labeled c-*erb*B-2 (A) and 36B4 (B) cDNA probes. The positions of molecular weight markers are shown to the left of the gels.

of c-*erb*B-2 mRNA; however, the 21MT-1 cells showed substantially higher levels of c-*erb*B-2 mRNA than those seen in the 21MT-2 cells (Figure 2A). Larger c-*erb*B-2 transcripts were also detected in the 21MT cell lines (Figure 2A), which is similar to previous results attained with other mammary carcinoma cell lines with c-*erb*B-2 gene amplification [12]. Reprobing of blots with the 36B4 probe confirmed equivalent loading of RNA in each lane (Figure 2B). Therefore, the H16N-2, 21MT-2, and 21MT-1 cell series showed a gradient of progressively increasing c-*erb*B-2 gene expression.

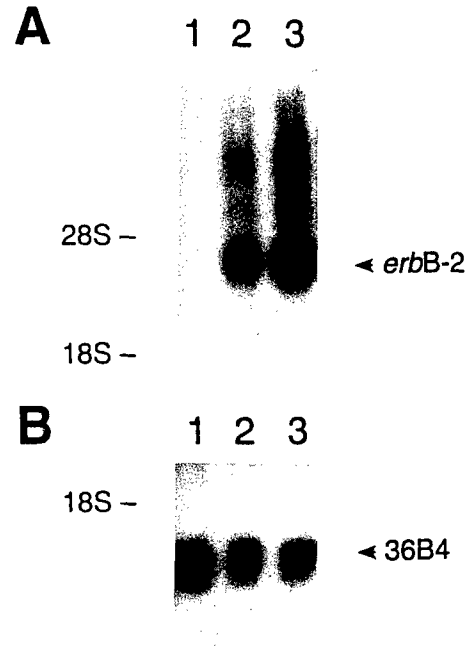

Figure 2. Progressive overexpression of c-*erb*B-2 in 21MT-2 and 21MT-1 cells in culture. Northern-blot analysis of c-*erb*B-2 gene expression in H16N-2 cells (lane 1), 21MT-2 cells (lane 2), and 21MT-1 cells (lane 3). Twenty micrograms of total RNA from each cell line was used to prepare northern blots hybridized with ^{32}P-labeled c-*erb*B-2 (A) and 36B4 (B) cDNA probes. The positions of 28S and 18S rRNAs are shown to the left of the gels.

The levels of p185^{erbB-2} protein and tyrosine phosphorylation in the cell lines were measured by anti-erbB-2 (Figure 3A) and anti-phosphotyrosine (Figure 3B) western blotting, respectively. The expression of unphosphorylated p185^{erbB-2} protein was detected in H16N-2 cell extracts on western blots after prolonged incubation with substrate or after immunoprecipitation of larger amounts of protein (data not shown). The 21MT-2 and 21MT-1 cells showed progressive increases in p185^{erbB-2} protein levels on blots loaded with equivalent amounts of membrane protein (Figure 3A). Furthermore, the 21MT cell lines showed concordant increases in the constitutive tyrosine phosphorylation of p185^{erbB-2} in cells cultured in serum-free medium devoid of exogenous growth factors (Figure 3B and C). Anti-erbB-2 immunoprecipitation was also performed and confirmed that the constitutively tyrosine-phosphorylated 185-kDa band was p185^{erbB-2} (Figure 3C). Thus, the amplification of c-*erb*B-2 in 21MT cells resulted in progressively elevated levels of p185^{erbB-2} with constitutive tyrosine kinase activity.

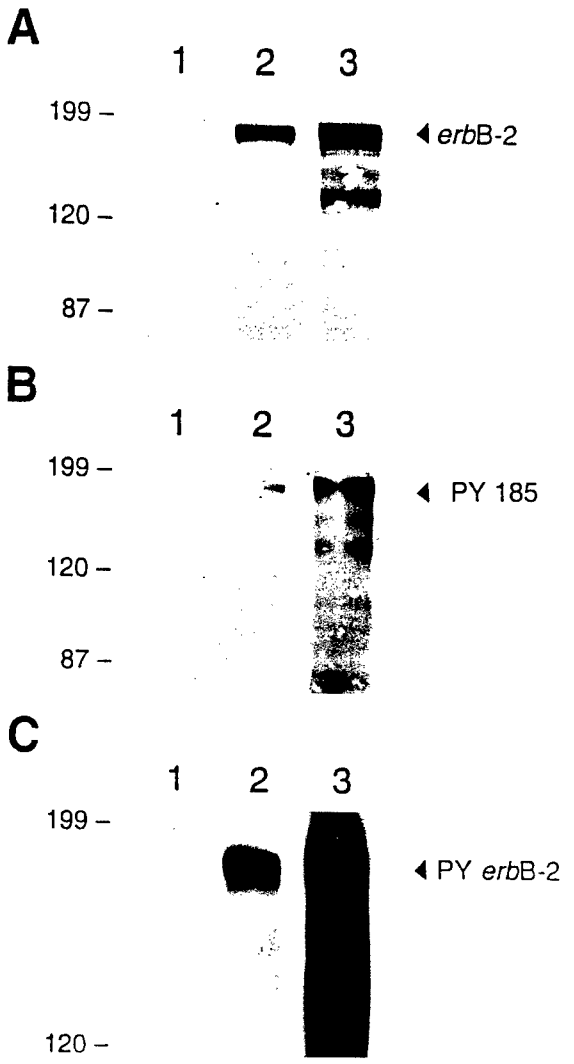

Figure 3. Overexpression of constitutively tyrosine-phosphorylated $p185^{erbB-2}$ in 21MT-2 and 21MT-1 cells in culture. Western-blot analysis of $p185^{erbB-2}$ and tyrosine-phosphorylated $p185^{erbB-2}$ in H16N-2 cells (lane 1), 21MT-2 cells (lane 2), and 21MT-1 cells (lane 3). Fifty micrograms of membrane protein from each cell line was used for SDS–polyacrylamide gel electrophoresis and immunoblotting with anti–erbB-2 (A) and anti-phosphotyrosine (B) antibodies. Cell lysates containing 2 mg of total cellular protein from each cell line were immunoprecipitated with anti–erbB-2 antibody and immunoblotted with anti-phosphotyrosine antibody (C).

Progressive Overexpression of c-*erb*B-2 in 21MT Cells Was Associated With IGF-Independent and Combined IGF- and EGF-Independent Proliferation in Culture

We developed a serum-free medium that allows the growth of the cell lines under fully defined conditions in culture. This allowed us to investigate the specific growth-factor requirements of these cell lines. The cell lines showed strikingly different growth-factor requirements when either insulin or EGF was removed from the culture medium (Figure 4). As we

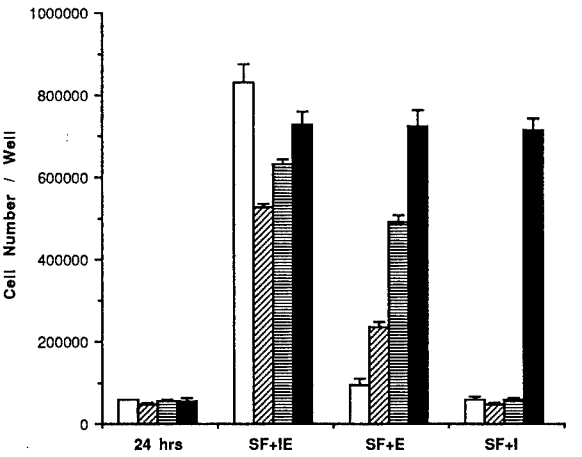

Figure 4. Progressive IGF and IGF/EGF independence of 21MT-2 and 21MT-1 cells in culture. Growth factor deletion experiments measuring the proliferation of MCF-10A cells (open bars), H16N-2 cells (diagonally hatched bars), 21MT-2 cells (horizontally hatched bars), and 21MT-1 cells (solid bars) by Coulter counting after 24 h and after an additional 9 d in serum-free (SF) medium containing both insulin and EGF (SF+IE), EGF only (SF+E), or insulin only (SF+I). The mean and standard deviation of triplicate samples for each condition are shown. The experiment shown is representative of the results of at least three separate experiments.

have previously shown [28,31], MCF-10A cells had a very strict requirement for both IGF-1 (or supraphysiological levels of insulin) and EGF under serum-free conditions in culture (Figure 4). H16N-2 cells exhibited similar growth-factor dependency but showed low levels of proliferation in the absence of insulin, probably because of the introduction of the HPV 16 genome into these cells [35]. In contrast, both 21MT-2 and 21MT-1 cells showed high levels of proliferation in culture in the complete absence of insulin, and 21MT-1 cells proliferated in the absence of EGF as well (Figure 4). 21MT-1 cells also proliferated when both insulin and EGF were omitted simultaneously from the culture medium (Figure 5). Therefore, 21MT-2 and 21MT-1 cells were IGF independent, whereas 21MT-1 cells were both IGF and EGF independent for growth in serum-free culture.

Insulin- or EGF-Like Autocrine Activity Was Not Detectable in 21MT Cell Cultures

To determine if IGFs, EGF-like factors, or both are released by 21MT cells, which may be acting in an autocrine fashion to stimulate their own growth in culture, conditioned medium experiments were performed by using MCF-10A cells as an indicator cell line (Figure 6). We previously showed that MCF-10A cells are an ideal indicator cell line for the detection of autocrine growth-factor activity that is released by carcinoma cells in culture because MCF-10A cells have a stringent requirement for both IGF and EGF and respond to physiological levels of these factors in culture [28,31]. As a positive control, we included

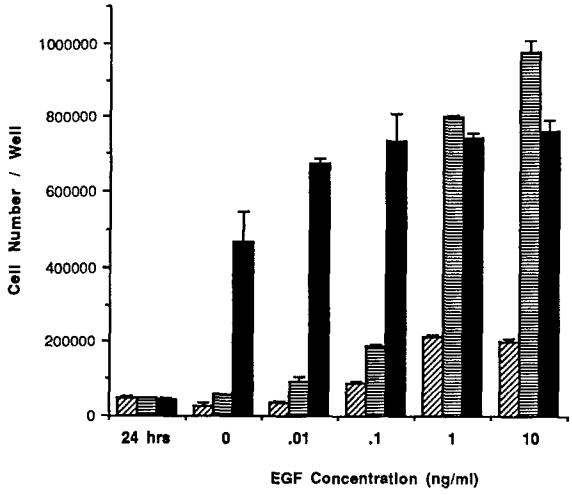

Figure 5. EGF dose response in the absence of insulin. H16N-2 cells (diagonally hatched bars), 21MT-2 cells (horizontally hatched bars), and 21MT-1 cells (solid bars) were cultured as in Figure 4 but with only the addition of EGF at various concentrations. The mean and standard deviation of triplicate samples for each condition are shown.

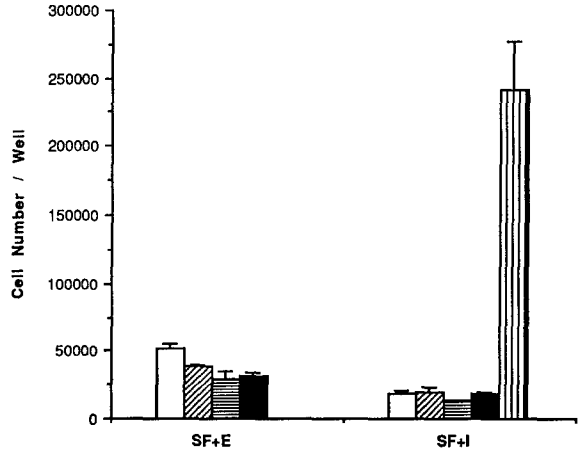

Figure 6. Insulin or EGF-like activity was not detectable in culture medium conditioned by H16N-2 or 21MT cell lines. Proliferation assay of MCF-10A cells cultured in serum-free medium without IGFs or EGF (open bars), or in IGF- and EGF-free media conditioned by H16N-2 cells (diagonally hatched bars), 21MT-2 cells (horizontally hatched bars), and 21MT-1 cells (solid bars), and 8-12 RMT carcinoma cells (vertical bars), which are known to secrete biologically active transforming growth factor α. The mean and standard deviation of triplicate samples for each condition are shown. The experiment shown is representative of the results of at least three separate experiments.

conditioned medium collected from 8-12 RMT rat carcinoma cells, which secrete EGF-like autocrine growth factors in culture [28]. We also previously showed that this assay detects IGF autocrine activity secreted by breast-cancer cells in culture [31]. By using this assay, no IGF- or EGF-like growth-stimulatory activity was detected in conditioned medium collected from 21MT-2 or 21MT-1 cells in culture (Figure 6).

To further test the possibility that cell-associated autocrine activity mediates the growth-factor independence of the 21MT cells, receptor-blocking experiments were performed with anti–IGF-1 receptor and anti-EGFR neutralizing antibodies that were previously shown to block the autocrine-mediated proliferation of certain human cancer cell lines in culture [47,48]. The addition of either antibody to cultures of MCF-10A cells grown in the presence of IGF-1 and EGF significantly inhibited their proliferation (Figure 7), confirming that these antibodies effectively blocked both IGF-1 receptor and EGFR responses in control cultures. However, neither of these antibodies inhibited the autonomous proliferation of 21MT-1 cells cultured in serum-free medium devoid of exogenous growth-factors (Figure 7). Anti–IGF-1 receptor antibodies also did not inhibit the proliferation of 21MT-2 cells cultured in medium devoid of IGF (data not shown). These data indicate that the growth factor–independent proliferation of 21MT cells is not due to the autocrine action of endogenous ligands for IGF-1 receptor or EGFR.

HRG Mimicked the Mitogenic Action of Both IGF and EGF in Mammary Epithelial Cells

To investigate the effects of $p185^{erbB-2}$ activation on the proliferation of the H16N-2, 21MT-2, and 21MT-1 cells, we studied the mitogenic effects of HRG [49,50] on cells under serum-free conditions and

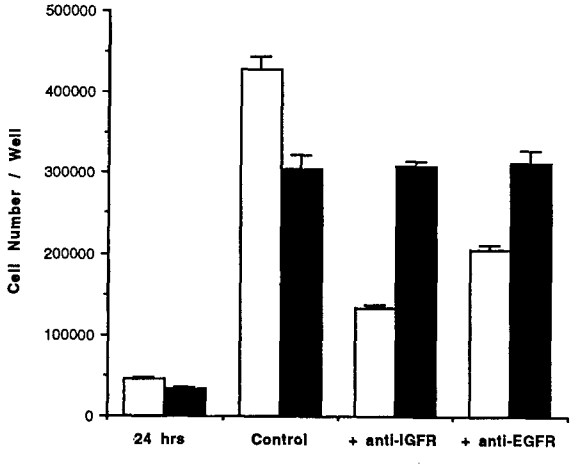

Figure 7. IGF or EGF-like autocrine activity was not detectable in 21MT cell cultures. Anti–IGF-1 receptor or anti-EGFR neutralizing antibodies (1 μg/mL) were added on day 1 to MCF-10A cells cultured in serum-free medium containing 10 ng/mL IGF-1 and 1 ng/mL EGF (open bars) and to 21MT-1 cells cultured without any IGF or EGF (solid bars). The cells were then counted 6 d later. All experiments were performed in triplicate, and the standard deviation of the mean is shown. The experiment shown is representative of the results of at least three separate experiments.

without insulin or EGF. HRGs are known to stimulate p185^{erbB-2} activation by direct binding to either erbB-3 or erbB-4 [36–39], and the expression of both p185^{erbB-2} and erbB-3 in genetically engineered systems leads to the formation of high-affinity binding sites for HRGs that induce tyrosine phosphorylation of both p185^{erbB-2} and *erb*B-3 [37,38]. We also recently found that the various isoforms of HRG are mitogenic for the non-neoplastic MCF-10A in the absence of either IGF or EGF and stimulate the tyrosine phosphorylation of p185^{erbB-2} in cells that co-express c-*erb*B-2 and c-*erb*B-3 but not c-*erb*B-4 [51]. Using PCR amplification of reverse-transcribed RNA, we found that H16N-2, 21MT-1, and 21MT-2 cells all expressed c-*erb*B-3 (Figure 8) but not c-*erb*B-4 (Figure 9), indicating that HRG could activate p185^{erbB-2} by binding to erbB-3 but not to erbB-4 in those cells. SK-BR-3 and T47D cells were included in these experiments as positive controls for the expression of c-*erb*B-3 and c-*erb*B-4, respectively. The use of RT-PCR to detect gene expression in these cells is ideal for effectively ruling out any role for erbB-4, because this technique is the most sensitive method for detecting very low levels of gene expression.

Stimulation of the H16N-2 cells with HRG potently induced proliferation when either insulin or EGF were removed from the culture medium (Figure 10). Therefore, as for MCF-10A cells, stimulation with HRG mimicked the combined mitogenic effects of either IGF and EGF in H16N-2 cells that expressed both c-*erb*B-2 and c-*erb*B-3 but not c-*erb*B-4. This ability of HRG to mimic the effects of both IGF and EGF is a unique property of this mitogen, which is unlike any of the other growth factors that we have previously tested [31]. The ligand-induced stimulation of p185^{erbB-2} and erbB-3 by HRG induced mammary epithelial-cell proliferation in the absence of IGF and EGF in a fashion similar to the growth factor–independent phenotypes seen in mammary carcinoma cells with high-level constitutive activation of p185^{erbB-2}. The 21MT-2 cells were also stimulated by HRG to some extent in the absence of insulin and to a great extent in the absence of EGF (Figure 10B). The 21MT-1 cells did not show mitogenic stimulation by HRG in the absence of insulin and only very slight stimulation in the absence of EGF (Figure 10C). Therefore, HRG did not induce additional mitogenic responses under conditions in which the 21MT cell lines already exhibited high-level growth-factor independence, and this reduced responsiveness to HRG was directly related to the level of a c-*erb*B-2 overexpression and the constitutive activation of p185^{erbB-2} in the 21MT cell lines.

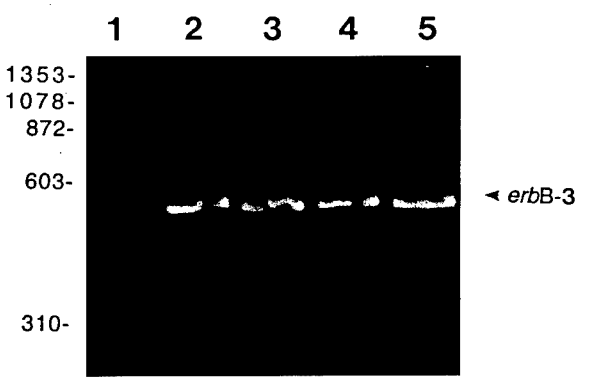

Figure 8. c-*erb*B-3 was expressed by H16N-2 and 21MT cells in culture. PCR amplification of a 550-bp fragment of the c-*erb*B-3 cDNA after RT of total RNA isolated from SK-BR-3 control cells (lanes 1 and 2), H16N-2 cells (lane 3), 21MT-2 cells (lane 4), and 21MT-1 cells (lane 5). Lane 1 shows a negative control reaction in which the RT step was omitted.

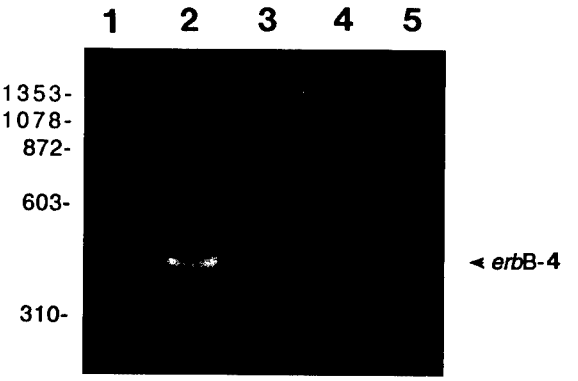

Figure 9. c-*erb*B-4 was not expressed by H16N-2 and 21MT cells in culture. PCR amplification of a 404-bp fragment of the c-*erb*B-4 cDNA after RT of total RNA isolated from T47D control cells (lanes 1 and 2), H16N-2 cells (lane 3), 21MT-2 cells (lane 4), and 21MT-1 cells (lane 5). Lane 1 shows a negative control reaction in which the RT step was omitted.

DISCUSSION

The findings reported here show that c-*erb*B-2 gene amplification and progressive overexpression of constitutively active p185^{erbB-2} in 21MT cell lines was associated with the attainment of IGF independence in 21MT-2 cells and both IGF and EGF independence in 21MT-1 cells. Therefore, the degree of growth-factor independence of mammary carcinoma cells with c-*erb*B-2 gene amplification may be a function of the level of c-*erb*B-2 overexpression, in that only IGF independence was seen at intermediate levels of p185^{erbB-2} activation in 21MT-2 cells, whereas both IGF and EGF independence was seen at the highest levels of p185^{erbB-2} activation in the 21MT-1 cells. Conditioned media and anti-receptor antibody blocking experiments showed that these growth abnormalities in 21MT cells were not due to the autocrine action of endogenous ligands for the IGF-1 receptor or EGFR. Furthermore, HRG, a ligand that activates p185^{erbB-2} by binding to erbB-3 or erbB-4 [36–39] potently stimulated the proliferation of H16N-2 cells (which expressed both c-*erb*B-2 and c-*erb*B-3 but not c-*erb*B-4) in the absence of either IGF or EGF in culture.

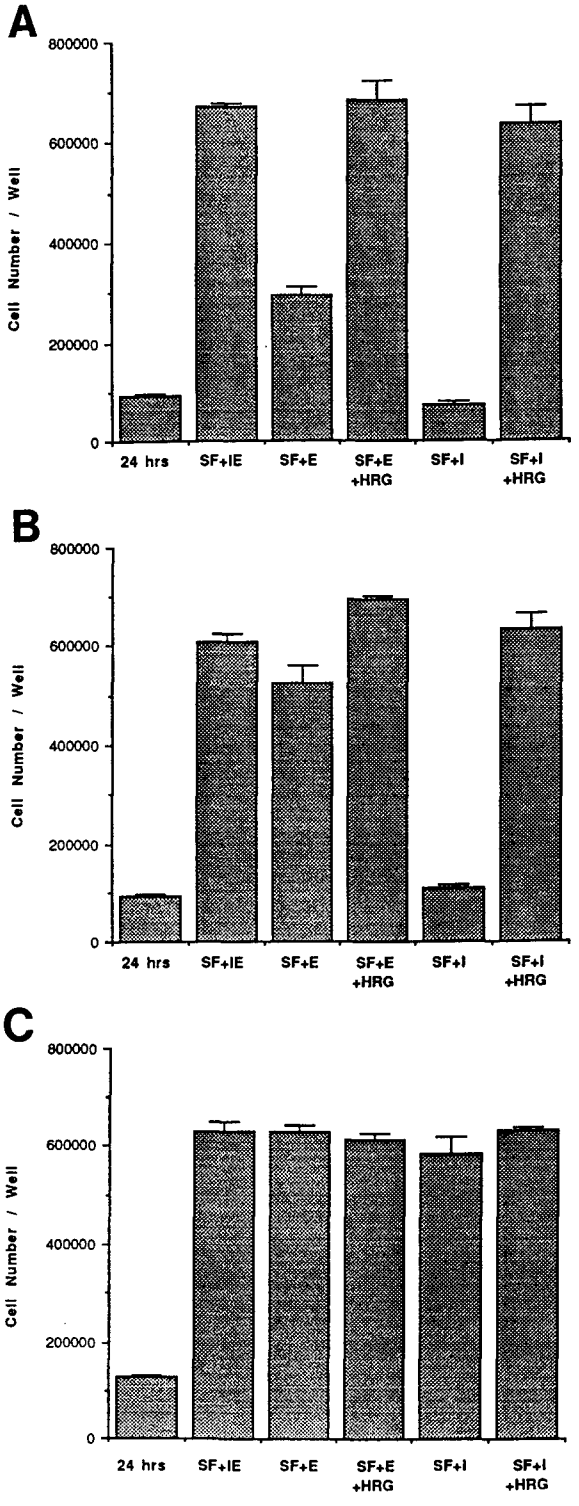

Figure 10. Mitogenic effects of HRG on H16N-2 and 21MT cells. H16N-2 cells (A), 21MT-2 cells (B), and 21MT-1 cells (C) were cultured in serum-free (SF) medium in the absence of either insulin (I) or EGF (E) and with or without 10 ng/mL HRG-β for 7d in culture. The mean and standard deviation of triplicate samples for each condition are shown. The experiment shown is representative of the results of at least three separate experiments.

Constitutively active $p185^{erbB-2}$ is known to effectively transform human mammary epithelial cells [52] and to directly stimulate various signal transduction pathways [53–55] that may normally require the presence of multiple growth factors. For example, the activated rat *neu* gene is known to constitutively activate phosphatidylinositol-3-kinase (PI3K) [55] and phospholipase C γ (PLCγ) [53]. Additionally, interactions between the different erbB receptors may be crucial for inducing the constitutive growth of c-*erb*B-2–overexpressing carcinoma cells. Interestingly, erbB-3 was previously found to be constitutively tyrosine-phosphorylated in c-*erb*B-2–overexpressing human mammary carcinoma cell lines [56], suggesting that the constitutive activation of erbB-3 may result from constitutive activation of $p185^{erbB-2}$ tyrosine kinase function and transactivation of erbB-3. Also, $p185^{erbB-2}$ and erbB-3 form high-affinity binding sites for HRGs through $p185^{erbB-2}$/erbB-3 heterodimer formation [37,38]. EGFR–erbB-3 chimeric receptors containing the cytoplasmic domain of erbB-3 are known to potently activate PI3K in response to stimulation of the chimeric receptor, whereas PI3K is only very weakly activated by wild-type EGFR in NIH/3T3 cells that express comparable levels of receptor [57]. This is consistent with sequence analysis of c-*erb*B-3, whose cytoplasmic domain contains multiple YXXM consensus sequences that, when tyrosine-phosphorylated, are known to form high-affinity binding sites for the 85-kDa regulatory subunit of PI3K [reviewed in 58]. PI3K is also known to be potently activated by insulin or IGF-1 receptors by direct association with the IRS-1 intermediary substrate [59,60] and PI3K activation is required for insulin-induced mitogenesis in culture [61]. Therefore, $p185^{erbB-2}$ homodimers, $p185^{erbB-2}$/erbB-3 heterodimers, or both may stimulate pathways otherwise stimulated by IGFs. EGF activation of PI3K is usually very weak, and recent evidence suggests that it occurs predominantly through EGFR/erbB-3 heterodimers [62,63].

Mammary carcinoma cells with c-*erb*B-2 gene amplification may also stimulate substrates such as PLCγ and ras GTPase-activating protein by the constitutive formation of $p185^{erbB-2}$ homodimers, $p185^{erbB-2}$/EGFR heterodimers, or both. Previously it was shown that overexpression of constitutively active wild-type $p185^{erbB-2}$ in CHO cells induces the recruitment and tyrosine phosphorylation of PLCγ (a substrate also stimulated by EGF) at very high levels of c-*erb*B-2 overexpression [53]. In addition, the recruitment of the Grb2 and SOS proteins by $p185^{erbB-2}$ was also recently shown to occur constitutively in mammary carcinoma cells with c-*erb*B-2 gene amplification, and the level of ras-mediated MAP kinase pathway activation was directly related to the level of c-*erb*B-2 overexpression and constitutive $p185^{erbB-2}$ tyrosine kinase activity [64]. The ras-mediated MAP kinase pathway is strongly stimulated by EGF [65] and has

recently been shown to be stimulated by HRG as well [66]. Furthermore, it has also been long known that $p185^{erbB-2}$ can form heterodimers with EGFR during its activation [67–69]. Thus, at high levels of c-erbB-2 overexpression, EGF independence may be induced by the constitutive activation of the PLCγ and ras GTPase-activating protein pathways involving $p185^{erbB-2}$ homodimers, $p185^{erbB-2}$/EGFR heterodimers, or both. In summary, these data show that constitutive $p185^{erbB-2}$ activation in tumor cells could feasibly stimulate both IGF- and EGF-dependent signal-transduction pathways within the cell, depending on the level of c-erbB-2 overexpression and on the extent of combined receptor-substrate associations.

The ability of various growth factors to stimulate normal cell growth was previously shown to fall into two groups termed competence factors (e.g., EGF) and progression factors (e.g., IGF-1) [reviewed in 70]. EGF is required during the early part of the G_1 phase of the cell cycle to make the cells competent for proliferation, whereas IGF is required near the end of G_1 for the cells to progress into S phase, and both EGF and IGF are required during the middle of G_1 [70]. Therefore, these different growth factors act synergistically to stimulate proliferation in a fashion that is not simply additive but is indicative of separate growth factor–responsive pathways that must be activated together for mitogenesis in untransformed cells that express normal levels of receptors. Interestingly, much of the work on how oncogene expression subverts these growth-factor requirements in tumor cells has emphasized responses associated with the requirement for competence factors, because other oncogenes (such as v-*erb*B, a truncated form of EGFR) were only implicated in the activation of pathways that normally require EGF for stimulation [71]. We propose that $p185^{erbB-2}$ constitutive activity in mammary carcinoma cells substitutes for growth factor–mediated signal transduction pathways that normally require both IGF and EGF and that the level of growth-factor independence depends on the level of $p185^{erbB-2}$ activation as well as the interaction of $p185^{erbB-2}$ with other erbB receptors. These properties of the erbB-2 protein may make it a particularly good target for the oncogenic conversion of cancer cells.

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